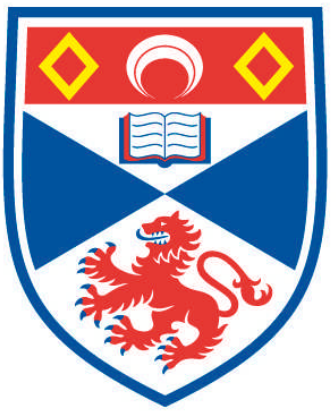
ASPECTS OF TISSUE MORPHOGENESIS AND ORGANISATION IN THE ZEBRA FISH, BRACHYDANIO RERIO

Paul Jeremy Dane

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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ASPECTS OF TISSUE MORPHOGENESIS AND ORGANISATION IN THE ZEBRA FISH

BRACHYDANIO RERIO

by

Paul Jeremy Dane

A thesis submitted for the degree of

Doctor of Philosophy

April 1986.

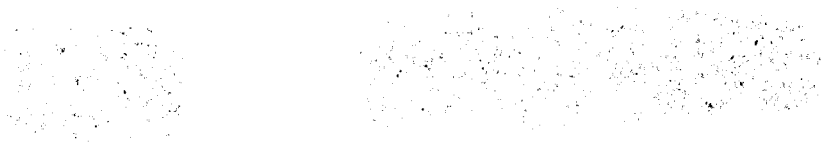
Department of Zoology
and Marine Biology,
University of St. Andrews.

UNIVERSITY CAREER

I entered the University of Leicester in September, 1980 and graduated with a first class Honours degree (Biological Sciences) in June, 1983. In October, 1983, I entered the Zoology Department at the University of St. Andrews, and began post-graduate research. The results of my work are presented here in this thesis for the degree of Doctor of Philosophy.

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Doctor of Philosophy.



April, 1986

DECLARATION

I Paul Jeremy Dane hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in September 1983, and as a candidate for the degree of Doctor of Philosophy in February, 1985.

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ABSTRACT

This thesis is mainly concerned with the impact of cellular mechanisms which influence a particular instance of tissue morphogenesis, namely development of the caudal fin of the zebra fish, Brachydanio rerio.

Ultrastructural analysis of fin fold morphogenesis in situ reveals that specific changes in epidermal cell shaping occur as a transient ectodermal ridge is generated. The ridge is converted to a fin fold by further changes in cell shaping which are spatio-temporally associated with the deposition of extracellular matrix material.

Microsurgical excisions of small portions of early folds support the suggestion that cell shape modulation and extracellular matrix organisation interact reciprocally during early fold formation. Studies using cytochalisin B show that actinoid microfilaments play an important role in generating the changes in epidermal cell shaping associated with fin fold morphogenesis. They also eliminate the possibility that overlying peridermis and epidermis which flank putative fin folds exert any great influence on the morphology of fold epidermis during apical ectodermal ridge generation. Furthermore, experiments employing tunicamycin indicate that cell adhesion and extracellular matrix deployment are particularly important in converting the apical ectodermal ridge into a fin fold and in subsequent stabilisation of the early fin fold. Microtubules do not appear to influence early fin fold morphogenesis although they are important during a later phase of fin fold expansion.

The spatial relationships between extracellular matrix orientation and cytoskeletal alignment in cell layers associated with the scales of certain teleosts have also been assessed. These studies have involved electron and immunufluorescence microscopy. They show that fibroblastic cells found at varying locations on the surfaces of scales display three types of microtubule arrays. Two of these arrays show intercellular alignment of microtubules which are spatially correlated with patterns of extracellular matrix deposition.

SUMMARY

This thesis is mainly concerned with the impact of cellular mechanisms which influence a particular instance of tissue morphogenesis, namely development of the caudal fin of the zebra fish, Brachydanio rerio.

Ultrastructural analysis of fin fold morphogenesis in situ reveals that specific changes in epidermal cell shaping occur as a transient ectodermal ridge is generated. Evidence has been obtained to support the argument that this ridge is similar in many ways to the apical ectodermal ridge of limb buds in higher vertebrates. The ridge is converted to a fin fold by further changes in cell shaping which are spatio-temporally associated with the deposition of extracellular matrix material. This material includes a set of previously undescribed extracellular cross fibres which span the central sub-epidermal space.

A tissue culture system for fin fold morphogenesis in excised tail buds was devised to assess the impact of a series of experimental perturbations on fin fold morphogenesis in vitro. Microsurgical excisions of small portions of early fin folds support the suggestion that cell shape modulation and extracellular matrix organisation interact reciprocally during early fold formation. Studies using cytochalasin B show that actinoid microfilaments play an important role in generating changes in epidermal cell shaping associated with fin fold morphogenesis. They also eliminate the possibility that overlying perdermis and epidermis which flanks putative fin folds

exert any great influence on the morphology of fold epidermis during apical ectodermal ridge generation. Futhermore, experiments employing tunicamycin indicate that cell adhesion and extracellular matrix deployment are particularly important in converting the apical ectodermal ridge into a fin fold and in subsequent stabilisation of the early fin fold. Perhaps surprisingly, early fin fold development is insensitive to nocodazole, hence microtubules do not appear to influence early fin fold morphogenesis, although they are important during a later phase of fin fold expansion.

The spatial relationships between extracellular matrix orientation and cytoskeletal alignment in cell layers associated with the scales of certain teleosts have also been assessed. These studies have involved electron and immunofluorescence microscopy. They show that fibroblastic cells found at varying locations on the surfaces of scales display three types of microtubule arrays. Two of these arrays show intercellular alignment of microtubules which are spatially correlated with patterns of extracellular matrix deposition. All three show previously unreported aspects of decentralised control of microtubule orientation and alignment.

CONTENTS

INTRODUCTION

This thesis is mainly concerned with an analysis of cellular mechanisms which influence tissue shaping during generation of the caudal fin fold in the teleost Brachydanio rerio.

1. CELLULAR MECHANISMS INVOLVED IN TISSUE MORPHOGENESIS

The cellular basis of tissue morphogenesis remains one of the central enigmas of developmental biology. A wide range of developing tissues and organs have been studied in which a number of different mechanisms have been shown to influence tissue morphology during embryogenesis; these are summarised below.

(a) Cell division and necrosis

Computer modelling of morphogenesis has shown that localised cell division can substantially alter the mechanical properties of a tissue and hence lead to changes in tissue morphology (Ransom & Matela, 1984). Oriented cell division may generate forces which operate in particular planes as observed during blastula development (Gustafson & Wolpert, 1967), brain development (Richman et al, 1975), and Drosophila clone organisation (Ransom, 1975; 1977). However, localised but unoriented division is more widespread and has been shown to play an important role in the morphogenesis of a number of tissues including the vertebrate limb bud (Ede & Law, 1964; Hornbruch & Wolpert, 1970; Searls & Janners, 1971; Lewis, 1975) and imaginal disc morphogenesis (Bryant & Schneiderman , 1964; Ransom & Matela, 1984).

Cell necrosis at specific sites in a developing tissue can lead to the formation of extracellular and extraembryonic spaces (for review see Hinchcliffe, 1982) as in the developing digits of the duck (Hurle & Fernandez-Teran, 1984) certain reptiles (Fallon & Cameron, 1977) and humans (Kelley, 1970).

(b) **The cytoskeleton**

Microtubules, microfilaments and intermediate filaments play and important role in the modulation and stabilisation of tissue shaping (for reviews see Burgess & Schroeder, 1979; Tucker, 1981).

Actinoid microfilaments often form extensive arrays beneath the cell surfaces of adjacent tissue cells. Contraction of these arrays may generate wedge- or flask-shaped cells which form folds or ridges within a flat sheet of tissue as, for example, during salivary gland morphogenesis (Spooner & Wessels, 1970; 1972; Spooner, 1973), gastrulation (Baker, 1965), neurulation (Karfunkel, 1974; Ostrovsky, Sanger & Lash, 1983), and lens invagination (Wrenn & Wessels, 1969) (for reviews see Wessels et al, 1971; Clarke & Spudich, 1977).

On the other hand, microfilament arrays may be involved in changes in cell morphology associated with cell migration (see Pollard & Weihung, 1974; Dunn & Heath, 1976). Microfilament contraction is thought to be co-ordinated between adjacent tissue cells as a result of the specific elastic properties of the filament arrays themselves (see Odell et al, 1981).

Microtubules are especially important in stabilising cell shape and often act in conjunction with microfilamentous arrays (for review see Tucker, 1981). Some of the most impressive examples of microtubule involvement in metazoan embryogenesis occur during axon outgrowth (Yamada, Spooner & Wessels, 1970; 1972; Solomon

1980), neurulation (Schroeder, 1970; Burnside, 1973; Karfunkel, 1974), sertoli cell morphogenesis (Fawcett, 1975; Vogl et al, 1983), and lens development (Byers & Porter, 1964).

Intermediate filaments are often considered to act as a form of "intracellular cellotape" which strengthens and stabilises cell morphology (for review see Lazarides, 1980). There are few tissues in which intermediate filaments are thought to play an active role in morphogenesis. However, several investigators have suggested that these fibres may play an important role in transmitting the forces generated by the modulation of other cytoskeletal proteins as, for example, during spermatogenesis (Vogl et al, 1983; Vogl, Linck & Dym, 1983).

(c) **Cell adhesion**

Tissue cells in vivo adhere to their neighbours and/or extracellular matrix material. Adhesivity may be modulated by the secretion of specific chemicals, for example, fibronectin and laminin (Yamada & Olden, 1978; Ekblom et al, 1980; Madri et al, 1980, Hynes, 1981; Kleinman, Klebe & Martin, 1981; England, 1982) or by changes in the physical properties of cells, for example, surface charge (see Garrod & Gingell, 1970; Lee, 1972; Schaefer & Brick, 1973). The correlation between variations in cell-cell adhesion and the sorting of different cell types in tissue culture has been widely studied (Steinberg, 1970; for reviews see Steinberg, 1978; Steinberg & Poole, 1981; 1982). However, it is extremely difficult to assess the impact of adhesivity modulation in cell shape changes which occur during tissue morphogenesis. There is much evidence that changes in cell adhesion observed in tissue culture can lead to changes in tissue morphology (see Phillips et al, 1977; Mittenthal

& Mazo, 1983), and increased substrate adhesion often leads to cell flattening (see Yamada, Yamada & Pastan, 1976). Whilst in vivo changes in cell adhesion may alter the overall mechanical properties of a tissue and so facilitate changes in tissue morphology as observed during ciliary body morphogenesis, (Bard & Ross, 1982), mammary gland development (Hogg, Harrison & Tickle, 1983), and limb budding (Heintzelman, Phillips & Davis, 1978).

(d) Cell migration

The migration of certain cells over other cells and extracellular matrices can be a direct consequence of changes in cell-substrate adhesion and provides a particularly important mechanism during morphogenesis of certain tissues, (for reviews, see Dowben & Shay, 1981; Wylie, Swan & Heasman, 1982).

Cells may migrate in isolation as, for example, during primordial germ cell migration, (Wylie, Bancroft & Heasman, 1976; Wylie & Heasman, 1976). Or they may migrate in groups or clusters (see Erickson, Tosney & Weston, 1980; Kolega, 1981), which can generate substantial changes in embryo morphology, for example, groups of cells which migrate from the vertebrate neural crest, eventually give rise to many body structures including Schwann cells, nerve cells and melanocytes (Hörstadius, 1950; Weston, 1970). On the other hand, cell migration may provide a mechanism whereby intact sheets of cells can be translocated within the embryonic or adult body. Good examples of this phenomenom are provided by teleost pigment cell migration (Trinkhaus, 1980), gastrulation (Keller, 1975)

and vertebrate wound healing (Radice, 1978; 1980). Cell migration plays an important role in teleost fin fold morphogenesis (Wood & Thorogood, 1984) at stages later than those described in this thesis.

(e) Extracellular matrix

The extracellular matrix provides an important substrate for metazoan cell adhesion, migration and morphogenesis. It consists of glycoprotein fibres, especially collagen, embedded in a matrix of proteoglycans and specialised proteins such as fibronectin (for review see Hay, 1983).

The secretion of specific extracellular molecules can facilitate cell migration (for review see Wylie, Swan & Heasman, 1982) as observed during neural crest cell migration where neural crest cells translocate along a 'pathway' which is rich in fibronectin and collagen (Löfberg, Ahlfors & Falström, 1978; 1980; Erickson, Tosney & Weston, 1980; Greenberg et al, 1981). There is also evidence that the pattern of extracellular matrix deposition may control branching morphology in a number of tissues, including the salivary gland (Grobstein & Cohen, 1965; Bernfield, Banerjee & Cohn 1972; Banerjee, Cohn & Bernfield,1977), mammary gland (Yang et al, 1979; Ormerod & Rudland, 1982), lung and ureteric bud (Wessels & Cohen, 1968) (for reviews see Bernfield, Cohn & Banerjee, 1973; Saxen, Ekblom & Thesleff, 1980).

(f) Cell shaping

Cell shape modulation is a major feature of tissue morphogenesis,

and has been shown to be particularly important during axon outgrowth (Yamada, Spooner & Wessels, 1970; 1971; Rakic, 1972). Cell shape changes result from many of the mechanisms described above (for reviews see Gospodarwicz, Greenberg & Birdwell, 1978; Gordon & jacobson, 1978). In many organs, cell flattening leads to tissue flattening as observed in studies of heart looping (Manasek, Burnside & Waterman, 1972) and may occur as a result of changes in cell adhesion, extracellular matrix composition and/or cytoskeletal deployment. The formation of wedge-shaped cells is often largely dependent on cytoskeletal modulation and can generate folds, ridges and thickenings in embryonic tissues as, for example, during neurulation (Karfunkel, 1974; Gordon & Jacobson, 1978) and lens invagination (Byers & Porter, 1964; Wrenn & Wessels, 1969). Cell shape changes may also occur as a result of, rather than be the cause of, tissue morphogenesis. For example, differential cell growth and division may lead to cell shape changes and tissue morphogenesis during thyroid development (Hilfer, 1973) and occular lens morphogenesis (Zwann & Hendrix, 1973).

(g) Positional information

Embryogenesis involves a number of tissue shape changes which are based on a range of different cellular mechanisms. These mechanisms must be spatio-temporally co-ordinated on a 'global' basis if a viable adult is to be formed. To achieve the correct pattern of development, tissue cells need to obtain information which relates to their position within the embryo (Wolpert, 1969; 1971; 1978;

Wolpert & Stein, 1984). Detailed studies of vertebrate limb regeneration have shown that every position within a particular tissue may be ascribed a positional value or "polar co-ordinate" (French, Bryant & Bryant, 1976; Bryant, Bryant & French, 1977). Polar co-ordinates may be evenly or unevenly distributed through the tissue and can provide a framework of information on which subsequent tissue morphogenesis can occur. The polar co-ordinate model has been applied to other developing tissues, for example, the vertebrate neural retina (MacDonald, 1977). One of the main schemes suggested for the supply of positional information is described below.

Specific regions within the embryo are thought to act as a transient source of morphogens which impart positional information. These morphogens may form a concentration gradient within the embryo as a reult of a number of possible cellular mechanisms (see Goodwin & Cohen, 1969; Wilby & Webster, 1970; Wolpert,Hincklin & Hornbruch, 1971). Tissue cells have a threshold sensitivity to these morphogens and where this threshold is exceeded, cells may develop a state appropriate to their position in the embryo (Wolpert, Hincklin & Hornbruch, 1971; Slack, 1976).

The possibility of gradient models has been particularly well explored in the developing chick limb bud where the interaction of chemical gradients from a number of sites is thought to help to specify the pattern of limb development (for review see Summerbell, Lewis & Wolpert, 1973; Summerbell & Lewis, 1975). This is of particular interest here as one of these sites, the apical ectodermal ridge, shows several morphological characteristics which are similar to the developing fin fold described in this thesis.

2. FIN FOLD MORPHOGENESIS

The earliest stages of fin fold morphogenesis involve the construction of a tissue with a relatively simple yet distinctive architecture. Peridermis and underlying epidermis form an elevated fin fold around a subepidermal space (Geraudie, 1977). This space contains well ordered arrays of extracellular fibres called actinotrichia (Bouvet, 1974). The fin folds of most of the teleost fins which have been examined develop from an apical ectodermal ridge-like structure (Bouvet, 1974; Geraudie & Françoise, 1973; Wood, 1982; Wood & Thorogood, 1984). The rapidity, precision and structural simplicity of fin fold morphogenesis make this an excellent structure for the study of the impact of cellular mechanisms on tissue morphogenesis.

Previous studies of teleost fin ultrastructure have mainly concentrated on morphogenetic stages which occur after the fin fold has been established (Geraudie & Françoise, 1973; Bouvet, 1974, Lanzing, 1976; Geraudie 1977; Geraudie & Landis, 1982). This thesis deals with mechanisms which are responsible for fin fold morphogenesis in the zebra fish, especially the specific changes in cell shaping and contact, as well as extracellular matrix secretion/ cytoskeletal deployment associated with the earliest stages of fin fold generation.

The following points are considered:-

1. Does cell shape modulation have a significant impact on overall fin fold morphogenesis, and are there any other mechanisms which are important in early fin fold development?

2. Is fin fold morphogenesis spatio-temporally confined to a specialised region of the embryonic tail bud?

3. What impact do the cytoskeleton and extracellular matrix have on cell shaping during fin fold morphogenesis?

The results of this experimental analysis show that there is a close functional correlation between cytoskeletal modulation and extracellular matrix deployment during fin fold morphogenesis. However, the developing fin fold, like most other embryonic tissues which have been studied in vivo (see Gorbsky & Borisy, 1985), is several cell layers thick and cannot be used for immunoflourescen visualisation of overall cytoskeletal organisation in relation to the distribution of extracellular matrix.

Fortunately, another tissue in Brachydanio provides an ideal opportunity to examine how the cytoskeleton may be spatially related to extracellular matrix orientation and microarchitecture. The tissue in question is the adult body scale. The opportunities it affords are considered below.

3. THE TELEOST SCALE

(a) Teleost scale morphology

Many aspects of the structure and ultrastructure of teleost scales and associated cells have been described previously (see Waterman, 1970; Lanzing & Wright, 1976). Each scale is a plate-like sheet of extracellular matrix material. An outer osseous layer covers a more interiorly situated fibrillary plate. This plate mainly consists of layers of collagen fibres stacked in an "orthogonal cross-ply" configuration (Bhatia, 1931; Cooke, 1967; Lanzing & Wright, 1976).

The osseous layer probably develops by ossification of the outer

levels of the fibrillary plate which is apparently effected by a layer of osteoblasts that covers a scale's outer osseous surface (Setna, 1934; Lanzing & Wright, 1976; Onozato & Watabe, 1979). The collagen fibres of the fibrillary plate are believed to be secreted by a layer of fibroblasts which cover a scale's inner fibrillary surface (Setna, 1934; Onozato & Watabe, 1979). These fibroblasts have been referred to as scleroblasts (see Neave, 1936) and hyposquamal cells (Waterman, 1970) by previous investigators, and will be referred to here as fibrillary plate fibroblasts or simply fibroblasts.

The osseous layer of each scale exhibits a patterns of ridges (circuli) and grooves (radii) which is highly variable in different teleost families, as is the overall shape of their scales. For example, in the salmonidae scales are roughly circular (cycloid) with concentric circuli or growth annulae, whereas in the cyprinidae, scales are irregularly shaped (ctenoid) with concentric arc shaped circuli and radially arranged radii (see Lagler & Bardach, 1962; Ricker, 1968).

(b) Co-alignment of cytoskeletal fibres and extracellular matrix in teleost scales

Recently, Byers, Fujiwara and Porter have used immunofluorescence microscopy to show that in the goldfish scale, fibroblast microtubules may be co-aligned with collagen fibres within the fibrillary plate (Byers, Fujiwara & Porter, 1980).

Chapter 7 of this thesis deals with an electron microscopic and immunoflourescence study of a range of different teleost scales, especially those of Brachydanio and explores the spatial correlation

between the cytoskeleton and extracellular matrix in the adult teleost scale. The following points are considered:-

1. Are there any cytoskeletal arrays other than those shown by Byers et al which are spatially correlated with the pattern of extracellular matrix distribution in the adult scale?

2. Are there correlations between the pattern of scale cell extracellular matrix deposition and scale cell cytoskeletal orientation at the ultrastructural level?

4. SELECTION OF MATERIAL

The zebra fish, Brachydanio rerio (Cyprinidae), has been used as the main experimental organism in the majority of this thesis. This teleost has already been widely studies (for review see Laale, 1977) as it is easy to maintain, and breeds readily and frequently in captivity. It produces large numbers of fertile eggs which are suitable for experimentation. It is therefore highly suitable for morphological and experimental analysis of tissue morphogenesis.

MATERIALS AND METHODS

1. FIN FOLD MORPHOGENESIS

(a) Maintenance and breeding of zebra fish (Brachydanio rerio)

Male and female zebra fish were isolated in separate stock tanks of fresh water at 27°C. Eggs were obtained by mating groups of 3 female fish with 6 male fish in a Legg trap (Axelrod et al, 1962) maintained at 29°C.

Fertilized eggs were removed from the trap and incubated in fresh water at 27°C. Stages in embryogenesis were distinguished using normal tables of development (Hisaoka & Battle, 1959).

(b) Tissue culture of isolated tail buds

A tissue culture systems was devised to allow in vitro experiments to be performed on developing tail buds. This system is described in detail in Chapter 2.

(c) Microsurgical manipulation of isolated tail buds in vitro

Isolated tail buds were subject to a variety of microsurgical manipulations. These are described in detail in Chapter 3.

(d) Drug studies

A variety of drugs were applied to cultured fin folds in an

attempt to asses the impact of cytoskeletal and extracellular matrix components on fin fold morphogenesis.

(i) **Cytochalasin B/nocodazole**

Separate stock solutions of 1mg of each drug (both from Sigma) in 1ml of 100% dimethyl sulphoxide (D.M.S.O.) were prepared. Stock solutions were diluted in culture medium to a variety of concentrations. The minimal effective dose of these drugs was assessed by incubating tail buds in 1ml culture medium at 27°C for 6 hours between 20 and 26 hours post fertilisation.* Cytochalasin B was found to be consistently effective at a concentration of 10µg/ml in 1% DMSO whereas nocodazole was used at 3µg/ml in 0.3% DMSO. A control culture of tail buds in sterile culture medium containing 1% DMSO was assessed.

(ii) **Tunicamycin**

Tunicamycin was applied to cultured tail buds in an identical way to cytochalasin B and nocodazole. This drug is, however, water soluble so stock solutions were prepared in sterile distilled water. They were then diluted to an effective dose of 5µg/ml in sterile culture medium.

Temporal variations in drug sensitivity were assessed by application of drugs at different times during fin fold morphogenesis (see Chapters 4, 5 & 6). After application of the drugs, samples were rinsed 10x in culture medium and left to recover in 1ml of culture medium for up to 25 hours at 27°C.

* Concentrations used in these assays were 0·1, 1·0, 3·0, 5·0, 10·0 and 100·0 µg/ml.

(e) **Light microscopy**

Isolated tail buds and tail bud cultures were placed on a glass slide in a drop of teleost phosphate buffered saline (P.B.S.) (Grimstone & Skaer, 1972) and mounted beneath glass coverslips. They were then examined and photographed with a Zeiss universal microscope using differential interference contrasts, bright field and dark field optics. Photographs were taken on Kodak Panatomic x film developed in Kodak D76 developer.

(f) **Electron microscopy**

(i) **Fixation**

(i.1) Glutaraldehyde/Osmium fixation

Routine fixation of samples was performed using a slight modification (50mM. phosphate buffer at pH 7.6) of a protocol devised by Tucker (1967).

(i.2) Osmium ferricyanide tannic acid uranium fixation (OsFeCN fixation)

Fixation with tannic acid has been used to enhance certain cellular components (McDonald, 1984). In this study a modification (1% tannic acid for 1 hour) of the protocol devised by McDonald (1984) was used to enhance contrast of extracellular matrix and membrane associated materials.

(ii) **Embedding**

Fixed samples were dehydrated in a graded ethanol series, immersed in propylene oxide and finally embedded in araldite resin.

(iii) **Microtomy/staining**

Embedded material was viewed with a Zeiss binocular dissecting microscope. Samples suitable for sectioning were mounted on araldite stubs and trimmed with a razor blade. "Thick" 1μm sections were taken with glass knives using an LKB ultratome III. They were then stained with methylene blue (Mullinger, 1964), and examined/photographed with a Zeiss universal microscope using bright field illumination. Thin sections were cut and picked up on uncoated copper grids which had been rinsed in glacial acetic acid, distilled water and acetone.

Thin sections were double stained with uranyl acetate (Gibbons & Grimstone, 1960) follwed by lead citrate (Reynolds, 1963). They were viewed and photographed with a Phillips EM 301 transmission electron microscope.

2. SCALES

(a) Culture of fish

Adult zebra fish (Brachydanio rerio) and neon tetras (Hyphessobrycon innesi) were purchased locally and maintained in a stock tank of fresh water at 27°C. Individual fish were removed for

experimentation. Six month old brown trout (Salmo trutta fario) were purchased from College Mill Trout Farm, Almondbank, Perthshire.

(b) **Isolation of scales**

Intact scales were isolated for microscopy using the following protocol. Fish were decapitated in a petri dish of fresh water. Strips of flanking ectoderm were removed using microsurgical scissors and placed in a drop of teleost P.B.S. on a clean microscope slide. Slides were inspected with a Zeiss binocular dissecting microscope and tungsten needles were used to prise scales from scale pouches. Isolated scales were stored in a drop of teleost P.B.S. and scale-free ectoderm was discarded.

(c) **Immunofluorescence microscopy**

Isolated scales were processed for anti-tubulin immunofluorescence using the following protocol.

Scales were fixed for 30 minutes at 20°C in 3% formaldehyde diluted with teleost P.B.S.. They were then rinsed 10x in P.B.S. and immersed in pre-cooled methanol. After incubation at -20°C for 10 minutes scales were re-warmed and re-hydrated by rinsing 5x in teleost P.B.S. at 20°C. They were incubated with a 1:10 dilution of primary antibody (rat anti-yeast tubulin YOL1/34, Serotec Ltd.) in P.B.S. for 1 hour at 37°C After 20 rinses in teleost P.B.S. scales were incubated in secondary antibody (1:20 dilution of FITC

conjugated sheep anti-rat IgG, Serotec Ltd) for 1 hour at 37°C in the dark. Finally, scales were rinsed 20x in teleost P.B.S. and mounted in 9:1 Glycerol:P.B.S.with 2mM Diazobicyclo octane (DABCO) (from Sigma) added to reduce the rate of photobleaching.

Scale preparations were viewed and photographed with a Leitz fluorescence microscope using fluorescence and phase contrast illumination. Kodak Plus X film was used and over developed (11 1/2 minutes at 20°C) in Kodak D76 developer to increase contrast and decrease exposure time.

(d) Differential interference microscopy

Isolated scales were mounted in a drop of P.B.S. on glass slides beneath glass coverslips. They were viewed and photographed with a Zeiss universal microscope using differential intereference contrast, bright and dark field optics. Kodak Panatomic X film was used and developed in Kodak D76 developer.

(e) Electron microscopy

(i) Decalcification/fixation

Teleost scales contain a layer of bony matrix which could not be sectioned using glass knives. A decalcification/ fixation protocol was devised. This protocol was broadly based on those described by Baird, Winbourne & Bockman (1967) and Waterman (1970). Sections of ectoderm containing scales were sandwiched between two pieces of fine copper mesh (approximately 1.50cm x 0.75cm, pore size 0.4mm)

to prevent them distorting during fixation and dehydration. They were then fixed in 4% glutaraldehyde in teleost P.B.S. for 4 hours at 0.5°C and decalcified in 4% glutaraldehyde which contained 0.1M EDTA in teleost P.B.S for 7 days at 0.5°C

Decalcification solution was replaced every 3 days. Samples were postfixed in 1-2% OsO_4 in teleost P.B.S. for 3.5 hours at 20°C before being rinsed 5x in P.B.S.

(ii) **Embedding, microtomy and staining**

Decalcified material was removed from the copper mesh and cut into small pieces with fine dissecting scissors prior to processing for electron microscopy as described in section 1(f) (ii) & (iii).

CHAPTER 1

EPIDERMAL MORPHOLOGY DURING FIN FOLD MORPHOGENESIS IN THE ZEBRA FISH (Brachydanio rerio)

INTRODUCTION

This chapter deals mainly with a light and transmission electron microscopical assessment of the impact of cell shaping and extracellular matrix deployment on generation of the caudal fin fold in the zebra fish (Brachydanio rerio). It provides a basis for the interpretation of experimental analyses of fin fold morphogenesis which is presented in subsequent chapters. Attention is drawn to certain similarities between fin fold morphogenesis and vertebrate limb bud development. Part of this work has been published previously, (Dane & Tucker, 1985).

RESULTS

The early tail bud consists of a mass of undifferentiated mesoderm covered by two layers of ectodermal cells (peridermis and epidermis) (Fig.13, arrow). The caudal fin fold develops from a narrow strip of ectoderm (8-10 cells wide) which extends around the distal tip of the tail bud.

The account that follows is based on examinations of dorsal portions of developing caudal fin folds. No marked changes in peridermal organisation were detected during early fin fold morphogenesis.

The main changes in epidermal cell organisation and extracellular matrix deployment within the subepidermal space are described below and summarised in Fig. 1.

(a) **Stage 19 (20-24h post fertilisation)**

The layer of cuboidal epidermal cells is overlain by a layer of partly flattened peridermal cells (Fig. 1A). Putative fin fold ectoderm does not exhibit any obvious differences in histological or fine structural organisation that distinguish it from tail bud ectoderm which is not destined to contribute to fin fold construction.

(b) **Stage 20 (24-27h post fertilisation)**

During stage 20 a ridge of ectoderm (approximately 30μm high) develops along the length of the tail bud. Three distinct developmental phases can be distinguished.

(i) Epidermal cells of the putative fin fold develop wedge-shaped cross-sectional profiles as they elongate and become 'centred' about an antero-posteriorly orientated axis on the mid-dorsal epidermal/mesodermal interface and group together to produce a local thickening of the ectoderm that forms an apical ectodermal ridge (Figs. 1B, 14). As a result of this change in cell shape the basal surfaces (those directed away from the peridermis) of these cells become reduced in area and lose contact with the underlying mesoderm (Figs. 1B, 14). Cross-sections of the ridge include 6-9 wedge shaped epidermal cells (Figs. 1B,14).

Fig. 1.

Schematic diagram showing the main changes in epidermal cell organisation and extracellular matrix deployment during fin fold morphogenesis.

(A) Stage 19. Peridermis (p), epidermis (e), mesoderm (m).

(B) Early stage 20. Wedge-shaped epidermal cells (w) become more widely separated from the underlying mesoderm (m).

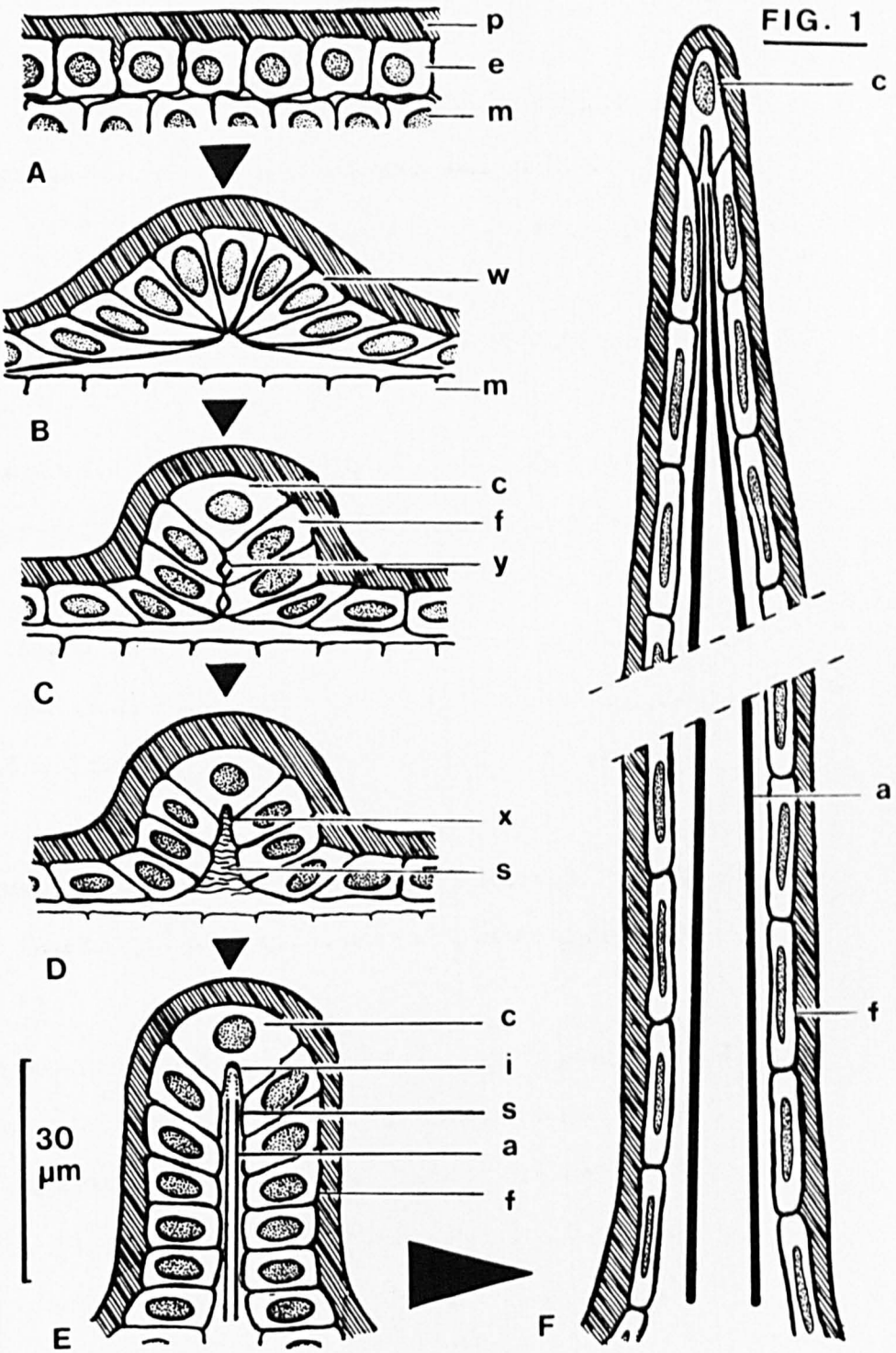
(C) Mid-stage 20. Cleft cells (c) are established at the apex of the ridge. Epidermal cells which flank the sides of the ridge (f) start to separate at several discrete loci (y).

(D) Early stage 21. A temporary set of extracellular cross fibres (x) span the newly formed subepidermal space (s).

(E) The number of epidermal cells (f) flanking the sides of the subepidermal space (s) begins to increase (compare with D). Actinotrichia (a) start to polymerise. Portions of actinotrichia located in the cleft-shaped invaginations (i) of cleft cells (c) are oriented parallel to the longitudinal axis of the fold.

(F) Stages 22-25. Most epidermal cells (f) flatten markedly. Cleft cells (c) still possess "clefts". The lengths and diameters of actinotrichia (a) increase.

FIG. 1



(ii) Serial transverse sections reveal that cells which form a single row running along the entire length of the apical ectodermal ridge retain their wedge-shaped cross-sectional profiles. These cells will be referred to as cleft cells. All other epidermal cells within a ridge become more or less rectangular in cross-section and form two closely apposed epidermal side walls of the ridge (Fig. 1C). As a consequence of this change in cell shaping the basal surfaces of epidermal cells in both side walls of a ridge increase in area. These surfaces are juxtaposed against the basal surfaces of other epidermal cells which contribute to the opposite side of a ridge.

(iii) A subepidermal space develops between the apposed basal surface of epidermal cells flanking the sides of a ridge. Cell surfaces separate at several discrete loci to begin with (Figs. 1C, 15) and separation is often associated with the production of cell surface invaginations (Fig. 16). The extracellular spaces that are created by cell surface separations gradually become more extensive and unite with each other until an uninterrupted extracellular subepidermal space runs between epidermal cells flanking the sides of the ridge which has now become a recognisable fin fold.

Each extracellular space generated during the production of a subepidermal space is lined by a basal lamina that is closely situated within 70nm of the cell membranes of separating epidermal cell surfaces (Figs.15,16). Dense bridges connect basal laminae to cell

membranes (Fig. 16) and strands of electron dense material run between basal laminae flanking either side of the developing subepidermal space (Fig. 16). Clusters of extracellular material which seem to represent a discontinuous basal lamina form at the epidermal-mesodermal interface adjacent to the subepidermal space (Fig. 15). This material is replaced by a continuous basal lamina during stage 21.

(c) Stage 21 (27-36h post fertilisation)

At the start of stage 21 the subepidermal space is a continuous extracellular cavity which extends from the base of the fin fold to the row of apical cleft cells (Fig. 17). Each cleft cell produces an invagination along the entire length of its narrow basal surface (Figs. 17,18) The ends of adjacent cleft cells overlap and in some cases interdigitate (Fig. 19). The invaginations of adjacent cleft cells are well aligned with each other and run as a continuous subepidermal space along the entire length of a cleft cell row. Cleft cells retain their invaginations at least until the end of stage 25 (Fig. 1). However, cells flanking the subepidermal space do not retain the cleft-like invaginations which form during initiation of the subepidermal space (Stage 20,iii). In regions where cleft-shaped invaginations of cleft cells or other epidermal cells have recently been formed, the invaginated cell membranes are associated with a cytoplasmic layer of dense granular material (up to 0.5μm thick) and small vesicles (see Fig. 16).

The number of cross-sectional epidermal cell profiles included

in a fin fold during early Stage 21 is the same (6-9) as those found in cross-sections of apical ectodermal ridges at Stage 20 prior to their conversion into a fin fold (compare Figs. 1B, C,D, 14, 17). During Stage 21, however, the fin fold expands considerably; it increases in height from about 30 to 100μm. As this increase occurs epidermal cells flanking the sides of the fin fold start to flatten and increase in number/cross section of a fin fold (compare Figs. 1, D & E).

At the start of Stage 21, a set of previously undescribed extracellular cross-fibres spans the subepidermal space (Fig. 1D). These fibres were readily apparent in the fin folds of tail buds fixed using the osmium ferricyanide fixation procedure (Fig. 20). They were difficult to detect when the more conventional fixation procedure was used (see Materials and Methods). In addition, a considerable amount of less highly organised extracellular material with a dense granular appearance ramifies between the cross-fibres and throughout most of the subepidermal space (Fig. 20). Where the cross-fibres approach the surfaces of epidermal cells their ends penetrate the basal lamina and apparently terminate in direct contact with cell surface membranes (Fig. 21). Cross-fibres are temporary components. They are numerous at the start of Stage 21 but become increasingly sparse as actinotrichia start to polymerise within the subepidermal space. Well defined cross-fibres spanning the space were not detected at later stages in morphogenesis.

Actinotrichia start to assemble during Stage 21 and increase in size during this and later stages. Throughout most of the fin fold actinotrichia form two distinct rows on either side of the sub-

epidermal space adjacent to the basal surfaces of flanking epidermal cells. These extracellular fibres are proximo-distally oriented in parallel arrays at right angles to the cross-fibres (Fig. 1E, 21). Actinotrichial arrangement at the distal border of the subepidermal space is different. Cross-sections of fin folds reveal cross-sectional profiles of actinotrichia within invaginations where cleft cells cap the subepidermal space (Fig. 18, arrows). This arrangement is in contrast to the longitudinal profiles obtained in more proximal regions of the subepidermal space. (Fig. 18). Examination of freshly excised tail buds using differential interference microscopy, and vertical thin sections cut in the plane of a fin fold, (Fig. 22) show that the distal extremities of actinotrichia curve in a posterior direction as they enter the distal portions of the subepidermal space contained within cleft cell invaginations.

Networks of intermediate filament bundles assemble in epidermal cells flanking the sides of the subepidermal space during Stage 21. These networks are highly localised. They are very closely juxtaposed against cell surface membranes facing the subepidermal space. Most of the bundles are positioned close to cell membrane regions directly beneath actinotrichia (Fig. 23, arrows).

(d) Stages 22-25 (36-96h post fertilisation)

During Stages 22-25 the fin fold continues to expand and forms a flattened blade like structure around the distal tip of the tail bud (Fig. 24). It increases in height from about 100-220μm. Concurrently, epidermal cells flanking the sides of the fold become

flattened compared with their more or less cuboidal shapes during the initial phases of Stage 21 (Fig. 27, compare Figs. 1E and F). These cells form a pavement epithelium flanking either side of the subepidermal space which increases considerably in width as well as height (Fig. 1F.)

As epidermal cells flatten an extensive system of intermediate filaments assembles into bundles which ramify throughout the cytoplasm of the fin fold epidermis (Figs. 25 & 26). A similar temporal relationship also occurs between intermediate filament assembly and peridermal cell flattening during earlier Stages (20 and 21). In both epidermal and peridermal cells, intermediate filament bundles are associated with densely staining bodies (up to 0.25μm in diameter) and with attachment desmosomes in regions where adjacent ectodermal cells make contact (Fig. 26).

Surprisingly few microtubules were detected in epidermal cells during apical ectodermal ridge formation and the early stages of fin fold construction. Actinoid stress fibres are usually sufficiently robust to be preserved by the fixation procedures employed. However, such fibres were not detected.

During Stages 22-25 actinotrichia increase in diameter (compare Figs. 23 and 25) but lose the antero-posteriorly curved portions that were present earlier. A basement lamella is interposed between actinotrichia and the basal lamina (Fig. 25) and mesodermal cells start to migrate into the subepidermal space in close proximity to actinotrichia (Fig. 27).

(e) 3-Dimensional analysis of epidermal cell dimensions during fin fold morphogenesis

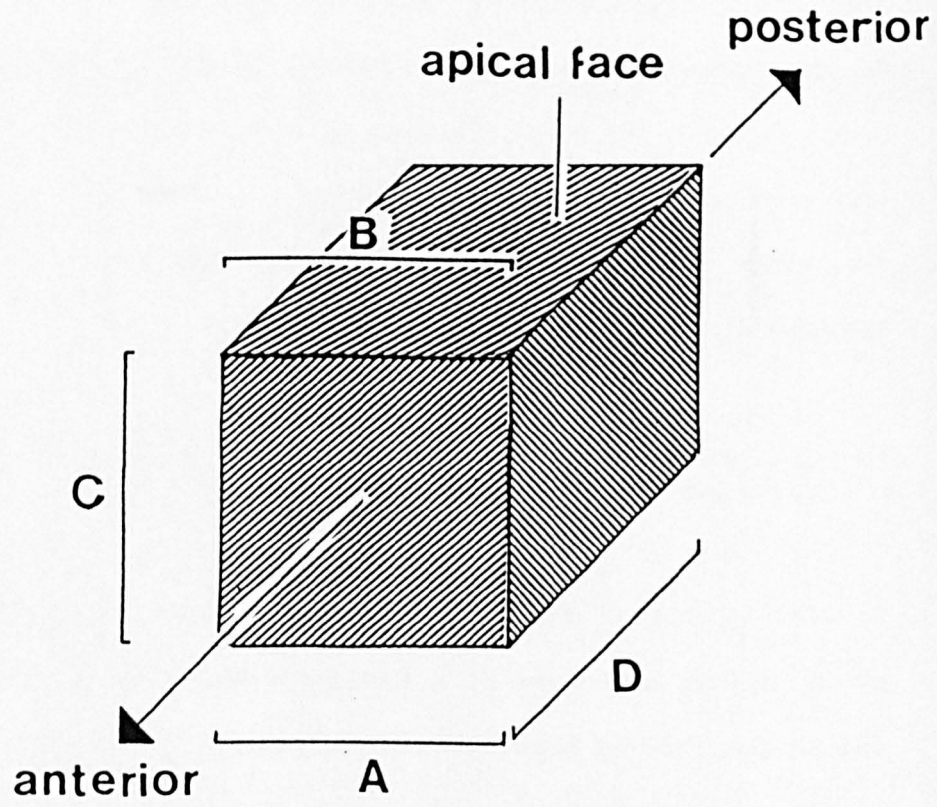
Electron micrographs provide a qualitative assessment of the changes in cell shaping which occur during fin fold morphogenesis. A quantitative analysis of changes in epidermal cell dimensions during fin fold morphogenesis was performed to ascertain whether the simple sequence of cell shape changes reported can give rise to the complex 3-dimensional structure of the fin fold (see Table 1 and Fig. 2). The results obtained show that epidermal cell wedging and elongation followed by flattening and shortening will lead to the generation of a 3-dimensional ridge-like structure which is subsequently converted to an extended fold.

(f) Changes in the number of cells involved in the different phases of fin fold morphogenesis

The number of cross-sectional profiles of epidermal cells contributing to the side walls of a fold increases from about 16 at the end of Stage 21 to about 40 during Stages 24 and 25. An analysis of the total number of epidermal cells involved in the different stages of fin fold morphogenesis shows that as the fold expands the number of cells within the fold increases (see Table 2 and Fig. 3). Hence an increase in the number of epidermal cells and cell flattening both contribute to growth of the fin fold during Stages 22 to 25. The increase in cell number might be due to epidermal cell proliferation within the fold and/or recruitment of adjacent epidermal cells into the fold.

Table I. Analysis of epidermal cell dimensions during fin fold morphogenesis

Light and transmission electron micrographs of longitudinal and transverse sections were used to assess the dimensions of putative fin fold epidermal cells at different stages in fold morphogenesis. The following conventions have been used in Table I:



A = Basal width.

B = Apical width.

C = Apical-Basal depth.

D = Anterior-Posterior length.

Table 1

STAGE	A (μm)	B (μm)	C (μm)	D (μm)
19 Putative fin fold epidermis	4.0 5.8 6.0 7.2 8.0 8.0 8.1 8.2 8.5 9.0	3.2 6.0 6.0 6.0 6.5 6.9 7.1 8.0 8.0 8.6	5.0 7.5 10.0 10.0 10.0 10.0 10.3 10.5 11.6 12.5	7.8 8.6 8.9 9.3 9.6 10.0 10.2 10.5 10.7 12.0
	Ave. = 7.3	Ave. = 6.6	Ave. = 9.7	Ave. = 9.8
20 Apical ecto-dermal ridge	- - - - - - - - - - - -	4.3 7.0 7.4 8.0 8.3 8.5 9.0 10.3 10.8 11.0	11.5 11.6 12.5 14.0 14.0 15.0 17.0 17.5 18.0 18.0	11.5 12.0 13.3 14.2 15.2 16.0 16.9 17.3 19.8 21.1
		Ave. = 8.5	Ave. = 14.9	Ave. = 15.7
21 Early fin fold	1.7 2.0 2.0 2.5 2.9 3.0 3.0 3.5 8.0 8.1	2.8 3.8 4.0 4.3 4.5 5.0 6.0 6.0 6.3 8.2	6.8 8.0 8.1 8.5 9.0 9.2 9.5 9.8 10.3 10.3	17.0 18.0 18.0 19.0 20.0 23.0 25.0 28.0 30.0 30.0
	Ave. = 3.7	Ave. = 5.1	Ave. = 8.9	Ave. = 22.8
23 expanded fin fold	12.0 13.0 14.0 16.0 16.0 17.0 18.0 18.0 20.0 22.0		2.0 2.5 2.5 3.0 3.0 3.5 3.5 4.0 4.0 4.5	8.0 9.0 9.5 10.0 10.0 10.0 10.5 10.5 11.0 11.5
	Ave. = 16.6		Ave. = 3.25	Ave. = 10.0

Average cell dimensions for each phase of morphogenesis have been used in the preparation of **Fig. 2**

Fig. 2

Schematic diagram showing how changes in cell dimensions may generate the three-dimensional changes in tissue shaping which occur during fin fold morphogenesis.

(1) Stage 19. Closely packed cuboidal putative fin fold epidermal cells contribute to a flattened sheet of epidermis.

(2) Stage 20. Putative fin fold epidermal cells become wedge-shaped and elongate to generate an elevated apical ectodermal ridge which contains approximately 7 cells in cross sectional profile.

(3) Stage 21. The basal faces of wedge-shaped epidermal cells increase in width as the apical ectodermal ridge is converted to a fin fold which contains a subepidermal space.

(4) Stage 22-25. Fin fold epidermis flattens apico-basally as the fin fold expands.

a = apical face

Dimensions taken from Table I.

FIGURE 2

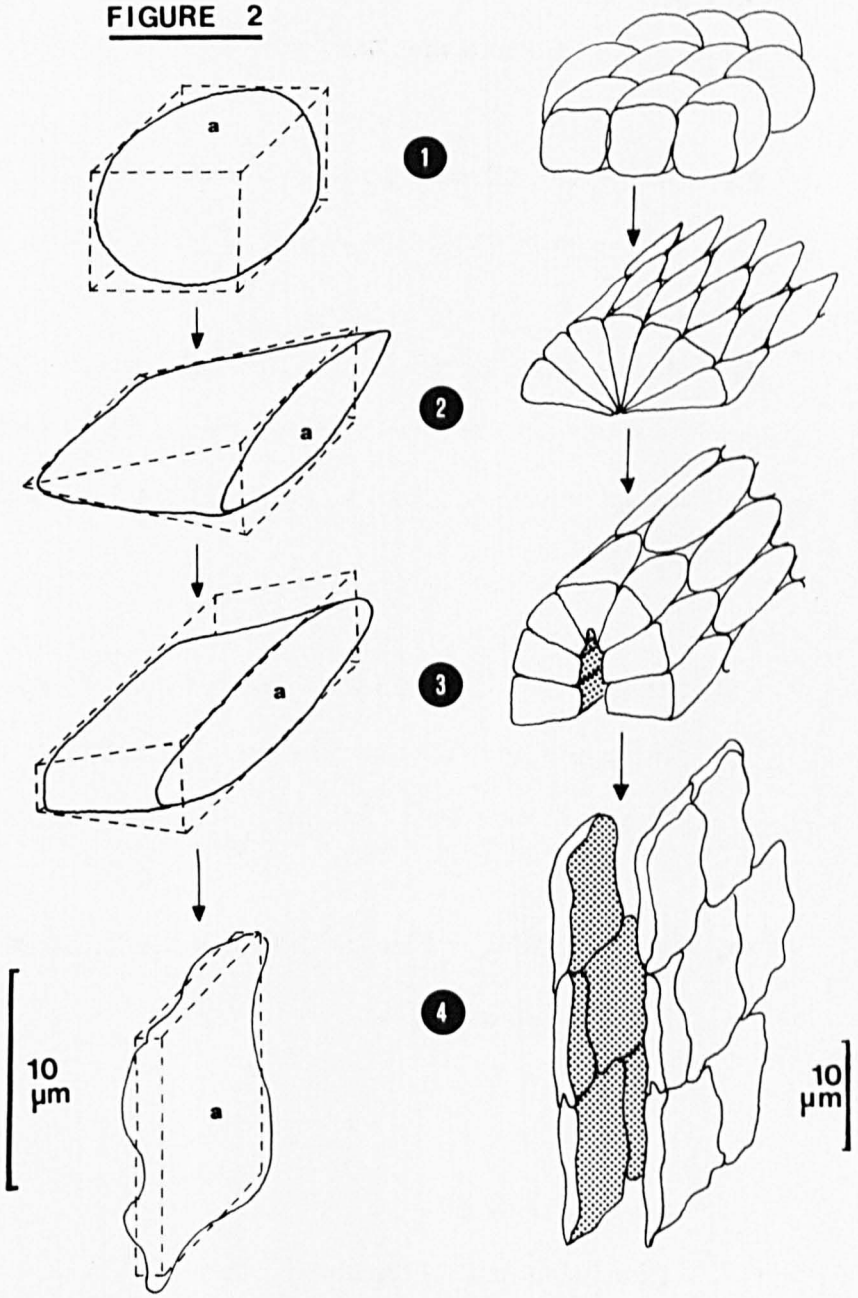


Table 2: Calculation of the total number of epidermal cells involved in constructing the fin fold

Approximate cell population sizes/fold were calculated in the following way.

(A) The average number of cells/cross sectional profile of a fold (A) was assessed from examinations of transmission electron micrographs.

(B) The average length of each cell in a fold (B) was measured from light micrographs of longitudinal methylene blue stained sections of folds.

(C) The percentage cell overlap of adjacent epidermal cells (C) was estimated from longitudinal sections of developing folds.

(D) Effective cell length (D) was calculated from:

$$D = \frac{100-C}{100} \quad B$$

(E) Total fold length of complete tail buds embedded in araldite resin (E) was measured.

(F) The approximate number of cells present in each fold (F) was calculated from:

$$F = \frac{EA}{D}$$

See **TABLE 2.**

Table 2

STAGE	A cells per cross section	B (μm)	C(%)	D(μm)	E(10^3μm)	F cells per fold
19 Putative fin fold epidermis	6 6 6 7 7 8 8 8 9 9 9	7.8 8.6 8.9 9.3 9.6 10.0 10.2 10.5 10.7 12.0	≈10	8.8	1.4 1.5 1.6 1.7 1.8 1.8 1.9 2.0	1.46 x 10^3
	Ave. = 7.5	Ave. = 9.8			Ave.= 1.71	
20 Apical ecto-dermal ridge	6 7 7 9 9 9 10 11 11 12	11.5 12.0 13.3 14.2 15.2 16.0 16.9 17.3 19.8 21.1	≈50	7.8	1.4 1.4 1.5 1.6 1.6 1.7 1.8 1.8	1.87 x 10^3
	Ave. = 9.1	Ave. = 15.7			Ave.= 1.6	
21 Early fin fold	7 8 8 8 9 9 10 10 11 12	17.0 18.0 18.0 19.0 20.0 23.0 25.0 28.0 30.0 30.0	≈40	13.7	1.6 1.6 1.6 1.7 1.8 1.8 2.0	1.14 x 10^3
	Ave. = 9.2	Ave. = 22.8			Ave.= 1.7	
23 Expanding fin fold	19 20 20 21 23 24 24 25 27 29	8.0 9.0 9.5 10.0 10.0 10.0 10.5 10.5 11.0 11.5	≈20	8.0	2.0 2.0 2.2 2.2 2.3 2.5 3.0	6.67 x 10^3
	Ave. = 23.2	Ave. = 10.0			Ave.= 2.3	

Calculated numbers of cells/folds (F) have been used in the presentation of the histograms **Fig 3.**

Fig. 3

Histogram showing how the total number of epidermal cells involved in fin fold construction varies during the different stages of morphogenesis (see **Table 2).** The total number of epidermal cells involved in the early phases of fold morphogenesis is relatively constant (see Epidermis, A.E.R., Stage 21 fin fold). However, as the fold starts to expand the total number of epidermal cells involved increases dramatically (Stage 23 fin fold).

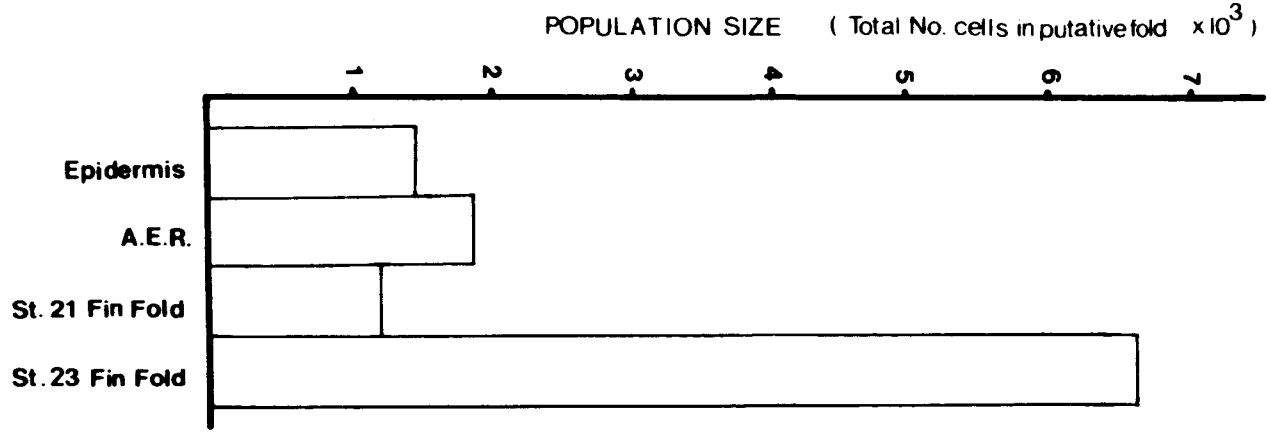

POPULATION SIZE (Total No. cells in putative fold x 10^3)
1
2
3
4
5
6
7
Epidermis
A.E.R.
St. 21 Fin Fold
St. 23 Fin Fold

FIGURE 3

& Bancroft, 1974; Goel & Mathur, 1977; Reynaud, Brabet & Adrian, 1979; Hurle & Fernandez-Teran, 1984; Todt & Fallon, 1984). It has been suggested that the notch in the chick wing bud apical ectodermal ridge may influence the positioning of distal wing elements (Todt & Fallon, 1984). An interesting comparison may be made between this role and specification of the orderly arrangement of actinotrichia (which help to define the position of bony fin rays (Geraudie & Landis, 1982)) in the subepidermal space of the fin fold.

(b) **Cell shape modulation**

Fin fold morphogenesis provides a clear example of a situation where spatiotemporal integration of individual cell shaping plays a major role in establishing overall tissue shape. This is particularly clear so far as the epidermis is concerned because cell number does not increase during the establishment of the main spatial characteristics of a fin fold, and the developing fin fold is less than 10 epidermal cells 'wide' during the period in question. Modulation of epidermal cell shaping reveals a considerable degree of spatial intricacy. For example, most epidermal cells exhibit a sequence of three distinct shape changes. All of these shape changes can be directly related to structural phases in the construction of a fin fold (Figs. 1 & 2). To begin with, more or less cuboidal cells adopt a wedge-shaped form and the 'wedges' are grouped together so that an apical ectodermal ridge is generated. Then these cells again become more or less cuboidal as they

form the side walls of the putative fold. Finally, cells flatten markedly as the blade of the fin fold expands. In contrast, one set of distinct cells, the apical row of cleft cells, undertake a different sequence of shape changes. These cells remain wedge-shaped when the others become cuboidal. They then develop a cleft-shaped invagination at their basal surface which persists as they start to flatten during fin fold expansion.

(c) Cleft cells

Cleft cells exhibit all the basic features of the other cells that have differentiated into fin fold epidermis. However, they are geographically distinct from the other epidermal cells by virtue of their apical positions and can also be distinguished by their 'morphological signature'. Cleft cells are apparent in micrographs of developing trout fin buds (Bouvet, 1974, Figs. 2,3; Geraudie, 1977, Fig. 2). The texts of these reports do not draw attention to these cells, although Geraudie (1977) mentions 'unstriated fibrils' which presumably correspond to the cross-sectional profiles of actinotrichia in the cleft cell invaginations reported here.

There is evidence that cleft cells may be functionally, as well as morphologically, distinct from other epidermal cells within the fin fold. For example, actinotrichia are differently oriented where they lie in the cleft-shaped invaginations of cleft cells with respect to their orientation elsewhere in the subepidermal space.

DISCUSSION

(a) The case for an apical ectodermal ridge

Similarities between early teleost fin folds and the apical ectodermal ridges of limb buds in higher vertebrates have been noted previously. For example, Wood (1982) describes killifish pectoral fin development as the modification of an apical ectodermal ridge to form a fin fold with a distinct subepidermal space. However, Geraudie (1978) uses the term 'pseudoapical ectodermal ridge' for the early fin fold in the trout pelvic fin on the grounds that it is "unlike the unfolded tetrapod apical ridge which is described as a thickening of the apical epidermis or a pseudostratified epithelium". There have been no previous ultrastructural studies of thin sections of the very earliest stages of fin morphogenesis to unequivocably resolve whether fin construction starts with an apical ectodermal ridge or with a fold. This examination of the morphogenesis of a fin establishes that to begin with there is a ridge-shaped epidermal thickening (which lacks a subepidermal space) as distinct from an epidermal fold.

In this context it is worth noting that the apical ectodermal ridges of some other vertebrate limb buds display a feature which is spatially homologous with one found during transition from ridge to fin fold. In these buds a small cleft-shaped indentation runs along the mid-basal line of each apical ectodermal ridge. Such indentations have been variously described as 'notches' and 'grooves' in a number of systems (Saunders, 1948; Jurand, 1965; Ede, Bellairs

(d) **Matrix organisation in the subepidermal space**

The dynamic and orderly layout of extracellular matrix fibres in the subepidermal space apparently provides a stable framework for support of the fin fold during early stages of morphogenesis. For example, at the start of Stage 21, numerous cross-fibres span the subepidermal space. These cross-fibres presumably operate as trusses by acting in conjunction with the substantial basal lamina which flanks the sides of the subepidermal space. By the time that cross-fibres depolymerise the actinotrichia have started to assemble and the cytoplasmic side walls of the subepidermal space are fortified by a juxtaposed meshwork of intermediate filaments, especially where they are subjacent to actinotrichia. Soon afterwards a basement lamella is laid down between the actinotrichia and cell surfaces flanking the subepidermal space. These components presumably take over the mechanical role of the cross-fibres. Elimination of cross-fibres is probably necessary to leave a clear path for the invasion of the subepidermal space by migrating mesodermal cells which are apparently guided into the space by actinotrichia (Wood & Thorogood, 1984). The posterior curving and overlap of the distal extremities of actinotrichia in the clefts of cleft cells probably contributes to maintenance of the structural integrity of the actinotrichial arrays and/or fin fold as a whole. Such overlap only occurs prior to formation of the basement lamella which may subsequently stabilise the arrangement of the closely associated actinotrichia.

(e) **Formation of the subepidermal space**

The subepidermal space is not produced by a straightforward folding of the epidermis. It results from the separation of the closely apposed basal surfaces of epidermal cells. In this respect, the procedure is similar to the development of ducts in mouse mammary glands. These ducts result from the fusion of extracellular spaces which open up within a solid mass of cells, apparently as a consequence of changes in cell adhesion and contact relationships (Hogg, Harrison & Tickle, 1983). It is unlike the formation of some other extracellular cavities such as the cavity inside most vertebrate neural tubes (see Karfunkel, 1974) and the lumen of the mammalian salivary gland (Spooner & Wessels, 1972) where epithelial sheets curve or fold to enclose pre-existing extracellular spaces. There is a similarity to neural tube production and salivary gland morphogenesis in terms of the involvement of wedge-shaped cells. However, contractile 'purse-string' arrays of microfilaments and associated belt-shaped attachment desmosomes at the 'thin ends' of wedges were not present during apical ectodermal ridge development.

(f) **Fin fold morphogenesis**

The most important feature to emerge from this investigation is evidence that several different procedures are substantially involved in fin fold generation. For example, there is a well-defined sequence of changes in epidermal cell shaping and surface contact relationships.

These apparently require alteration in both cytoskeletal organisation and cell surface adhesion. The shaping sequence is complemented by intricate modulations in the layout of extracellular matrix fibres in the subepidermal space. This emphasises the possibility that the dynamics of actinotrichial and cross-fibre deployment are important for stabilizing and promoting new aspects of epidermal architecture. The relationship between cleft cells, the subepidermal space and curved portions of actinotrichia may indicate that this "special" row of cells plays a key role in co-ordinating the pattern of intra- and extra-cellular events. Subsequent chapters attempt to experimentally assess the impact of cell shape changes, cyto-skeletal modulation and extracellular matrix deployment on fold morphogenesis and to elucidate how integration of their contributions may be controlled.

CHAPTER 2

ANALYSIS OF FIN FOLD MORPHOGENESIS IN ISOLATED TAIL BUDS CULTURED IN VITRO

INTRODUCTION

This chapter deals with an in vitro culture system which was devised to permit experimental analysis of fin fold morphogenesis. Whole embryos were not suitable for experimental analysis because they had very little mechanical strength and were extremely difficult to manipulate. Therefore, a protocol was devised for culturing isolated tail buds and associated fin folds in vitro. The question of whether fin folds which had been isolated and cultured in vitro were suitable for experimental analysis is discussed.

MATERIALS AND METHODS

(a) Sterilisation of embryos

A variety of sterilisation techniques were tried to prevent contamination of tail bud cultures. Initially, fertilised eggs were incubated in 10ml sterilised bottles which contained 1ml of sterile distilled water with 100 units/ml Penecillin and 100µg/ml Streptomycin added. This protocol was not successful because high concentrations of antibiotics dramatically increased the rate of embryo fatality. Absolute ethanol, 70% ethanol and calcium hypochlorite solution (Milner and Sang, 1974) were each used to surface sterilise

egg shell membranes prior to dissection. Again these protocols led to embryo necrosis. Healthy sterile embryos which were suitable for dissection were obtained by rinsing fertilised eggs 5 times in sterile distilled water before dissection.

(b) **Isolation of tail buds from embryos**

Appropriately staged sterile embryos were inspected using a Zeiss binocular dissecting microscope and placed on a sterile siliconised microscope slide in a drop of sterile phosphate buffered teleost saline (teleost P.B.S.) (66mM, pH 7.6) (Grimstone & Skaer, 1972). Embryos were then teased from their egg shell membranes and cut at the posterior end of the yolk sac using a pair of fine tungsten needles. The posterior portion of each embryo which contained an intact tail bud and developing fin fold was transferred to a fresh drop of teleost P.B.S. The remainder of the embryo was discarded.

(c) **Tissue culture of isolated tail buds**

Sterile isolated tail buds were cultured in the following medium:

Minimal essential medium (Eagles) with HEPES. (Flow Laboratories)

10% Foetal calf serum. (Flow Laboratories)

5mM Glutamine. (Flow Laboratories)

1% Kanamycin. (Gibco)

Isolated tail buds were rinsed three times in separate drops of sterile culture medium on a siliconised microscope slide before they were transferred to sterile bottles which each contained 1ml of culture medium at 27°C. Developing tail buds were maintained for up to 8 days *in vitro*.

RESULTS

(a) Tail buds cultured for 1-3 days *in vitro*

Tail buds which are isolated from Stage 19 embryos develop normally for up to 3 days *in vitro*. This period is equivalent to the total time which the embryo spends in the egg *in vivo* (total incubation time).

Fin fold morphogenesis proceeds slightly less rapidly *in vitro* than *in vivo*: Folds which are cultured for 24 hours *in vitro* from Stage 19 embryos (44 hours total incubation time) are morphologically identical to Stage 22 folds grown *in vivo* (37-40 hours total incubation time) (compare Figs. 24 & 28). Putative fin fold ectodermal cells within isolated tail buds undergo the normal sequence of shape changes. Actinotrichia form parallel arrays (Fig. 29) which curve posteriorly to run proximo-distally within the cleft cell invagination (Fig. 30).

(b) **Tail buds cultured for 4-8 days in vitro**

Tail buds which are cultured for more than 4 days in vitro develop morphological abnormalities. They often become contorted (Fig. 31) and actinotrichia within the fin folds become disoriented (Fig. 32).

DISCUSSION

Tissue culture of isolated tail buds

These results indicate that the culture system described above is suitable for use in experimental analyses of fin fold morphogenesis throughout the period of development described in Chapter 1. Isolated tail buds which have been cultured for up to 3 days in vitro are morphologically identical to the tail buds of embryos grown to the pre-hatch stage (Stage 25) in vivo.

Teleost embryogenesis is temperature dependent (Bouvet, 1974). It is likely that the slight retardation of morphogenesis in vitro reported here is due to embryos cooling to room temperature (around 19°C) during sterilisation and dissection (aquaria and cultures were maintained at 27°C).

Tail buds which have been cultured for more than 4 days do not elongate as much as tail buds in situ. T. Elsdale, (M.R.C. Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh) has found that if ectoderm from anterior portions

of the embryo is left attached to isolated frog tail buds in culture then there is an increase in the probability that these tail buds will extend in a similar way to that which occurs in situ, (personal communication). This may indicate that anterior ectoderm is recruited into the extending frog tail bud. Similar events may occur during tail bud extension in the zebra fish. Alternatively, nutrient deficiency and/or build up of waste products may inhibit normal morphogenesis in 4-8 day old cultures.

CHAPTER 3

MICROSURGICAL ANALYSIS OF FIN FOLD MORPHOGENESIS IN ISOLATED TAIL BUDS CULTURED IN VITRO

INTRODUCTION

This chapter deals with a series of microsurgical experiments which were devised to assess the regenerative capabilities of the fin fold and the importance of different regions of the early fin fold in overall fin fold morphogenesis.

MATERIALS AND METHODS

Microsurgical manipulation of isolated tail buds cultured in vitro

Isolated tail buds were subject to a variety of microsurgical manipulations. Twenty five hour-old tail buds were placed on sterile siliconised glass slides in drops of sterile culture medium. Portions of developing fin folds (see below) were then removed with fine tungsten needles:

1. Fin fold and underlying mesoderm - the tip of the tail bud was amputated (25 samples taken).
2. Entire fin fold - a needle was inserted into the base of the fin fold and swiftly moved posteriorly (11 samples taken).
3. Distal fin fold - a needle was inserted into the middle of the fold and swiftly moved posteriorly. (16 samples taken).

4. Central portion of a fin fold - 2 needles were inserted into the middle of a fold, one was moved anteriorly, the other was moved posteriorly (8 samples taken).

Once microsurgery had been completed, isolated tail buds were left to recover in sterile tissue culture medium as described in Chapter 2.

RESULTS

Initial attempts to remove portions of the developing apical ectodermal ridge were unsuccessful. The early tail bud is extremely sensitive and application of force to any specific area causes the entire structure to collapse. However, it is possible to remove the portions of the early fin fold described above. The main types of microsurgical interventions and their consequences are summarised in Fig. 4.

(a) Removal of distal portions of a fin fold

When distal portions of a fin fold (including part of the cleft cell row) are removed they are not replaced. (Compare Figs. 33 & 34). Thin sections indicate that damage to the distal end of the fold causes the subepidermal space to swell and form a balloon-like cavity (Figs. 4B, 35). Actinotrichia are disoriented and the migration of mesodermal cells is retarded (Fig. 35). The apex of each balloon-like fold is capped by ectodermal cells which contain numerous invaginations (Figs. 4B, 36), rather than

Fig. 4

Schematic diagram showing the impact of various microsurgical interventions on subsequent fin fold morphogenesis. Amputated regions are shown by shading.

(A) Control cultures undergo the normal sequence of morphogenetic changes for up to 72 hours in culture.

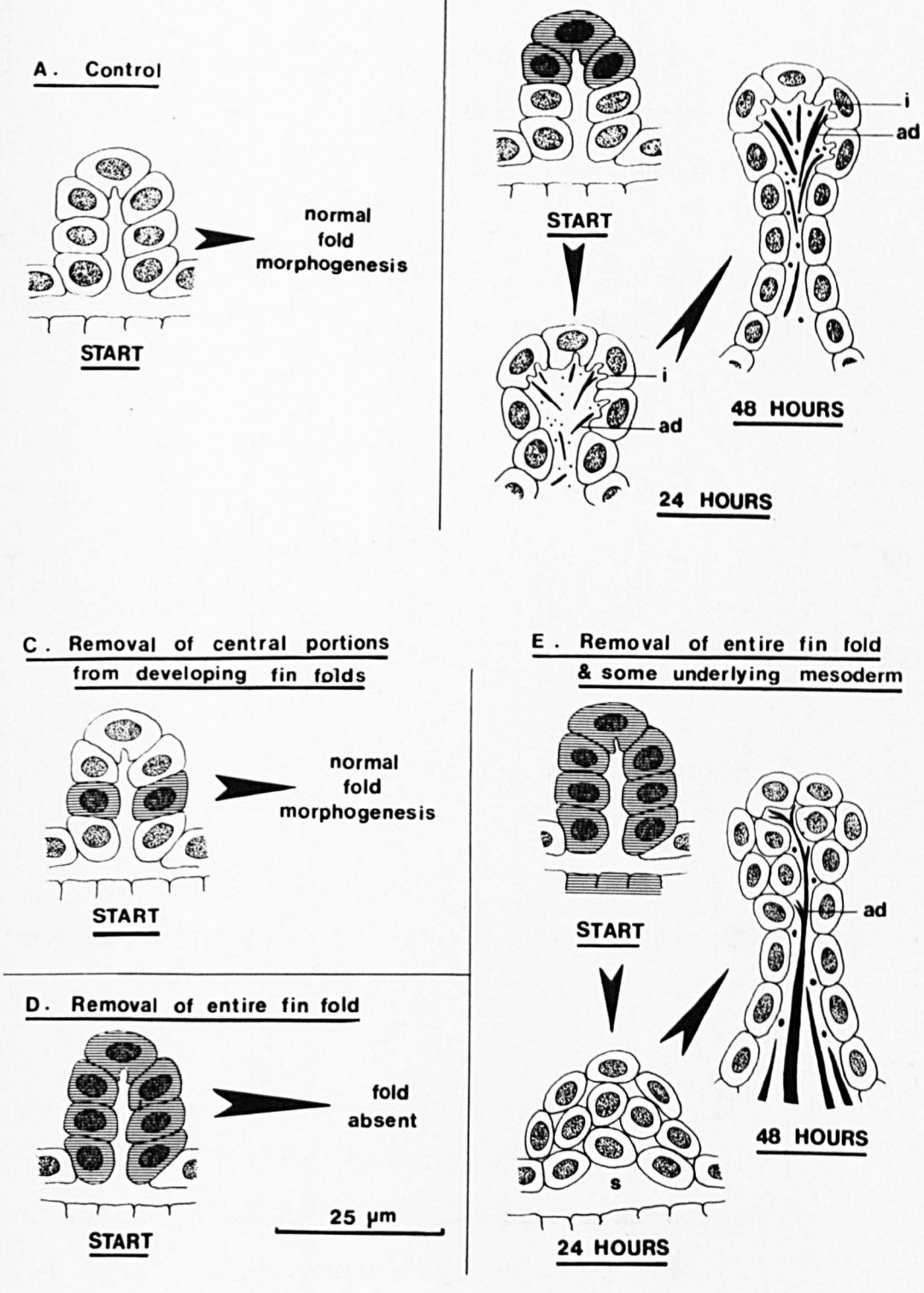
(B) Removal of an apical portion of the fold generates an enlarged 'balloon-like' subepidermal space which contains disoriented actinotrichia (ad). The basal faces of epidermal cells towards the apex of these folds contain numerous surface invaginations (i).

(C) Removal of central portions of the fold does not alter subsequent morphogenesis.

(D) Removal of the entire fin fold prevents subsequent fin fold morphogenesis.

(E) Removal of the entire fin fold and some underlying mesoderm leads to the generation of a wound blastema. Within 24 hours a small extracellular space (s) develops at the base of the blastema. This space then enlarges and contains disoriented actinotrichia (ad).

FIGURE 4


A. Control
normal
fold
morphogenesis
START
B. Removal of apical portions
from developing fin folds
START
i
ad
48 HOURS
i
ad
24 HOURS
C. Removal of central portions
from developing fin folds
normal
fold
morphogenesis
START
D. Removal of entire fin fold
fold
absent
25 µm
START
E. Removal of entire fin fold
& some underlying mesoderm
START
ad
48 HOURS
s
24 HOURS

the normal single row of cleft cells which is found in control cultures.

(b) **Removal of central portions of the fold**

The pattern of cell shaping and extracellular matrix deployment involved in fin fold morphogenesis is not altered when central portions of the fold are removed. Damaged sites are covered by ectodermal cells within 3 minutes of the operation (Fig. 4C).

(c) **Removal of a complete fin fold**

Fin folds do not regenerate when the entire fold is removed from a tail bud in which the mesoderm has been left intact (Figs. 4D, 37).

(d) **Removal of a complete fin fold and underlying mesoderm**

When portions of the fin fold ectoderm and some underlying mesoderm are removed, an abnormal fin fold regenerates from a wound blastema (Figs. 4E, 38, 39). Thin sections indicate that the subepidermal space in these regenerating folds develops from a cavity which contains granular extracellular matrix, at the base of the blastema (Figs. 4E, 40) This cavity enlarges distally (Fig. 41) until it eventually forms a contorted subepidermal space which contains disoriented actinotrichia (Figs. 4E, 42). Apical portions of actinotrichia terminate between the cell membranes of adjacent

ectodermal cells rather that in a cleft cell invagination. Ectodermal cells which form the side walls of the regenerating fold are highly disorganised and the row of cleft cells does not regenerate after it has been removed.

DISCUSSION

(a) The effect of surgical trauma on fin fold morphogenesis *in vitro*

Experiments which involve microsurgical manipulation are bound to impose a certain degree of trauma on the developing tail bud. The extent to which abnormalities in the form of tail buds and fin folds are induced solely by such trauma and not by the experimental peturbations can not be fully assessed. However, the fact that isolated tail buds do develop normal fin folds after excision of central portions of the fold indicates that any response to trauma does not significantly alter fin fold morphogenesis *in vitro*.

(b) Regenerative ability of the fin fold

Regeneration of body parts has been reported in a wide variety of organisms (for review see Kiortsis & Trumpusch, 1965; Bryant, Bryant & French, 1977). Adult teleosts also show regenerative ability, for example, *Tilapia* (Kemp & Park, 1970), *Carassius* (Kemp *et al*, 1978), *Stenopus* (Morgan, 1902), *Gambussia* (Sichel, 1965) and *Brachydanio* (Manner & Ekandem, 1973) all regenerate body fins after removal of part or whole adult fins which contain mesoderm, actinotrichia and lepidotrichia.

This initial study of the regenerative capabilities of an embryonic teleost at the first stages of fin fold morphogenesis indicates

that the fin fold will not regenerate unless both ectoderm and part of the mesoderm have been removed. This finding corresponds with evidence that mesodermal cells are important in blastema formation (Hay, 1959; Thornton, 1959; Goss, 1965) and subsequent regeneration (Goss, 1965; Faber, 1965; Saunders & Reus, 1974; Iten & Bryant, 1975; Stocum, 1977). It is interesting to note that similar ectodermal-mesodermal interactions are important in early limb development (Zwilling, 1964; Saunders & Gasseling, 1968; Saunders, 1972; Maccabe & Richardson, 1982; for review see Ede, Hinchcliffe & Balls, 1977) they may also be involved in establishment of the teleost apical ectodermal ridge.

The orderly sequence of extracellular matrix and cell shape modulation reported in Chapter 1 almost certainly plays a crucial role in morphogenesis of the early fin fold and its subsequent conversion to an adult fold. This sequence is not repeated after microsurgical intervention. Therefore, when post-microsurgical regeneration occurs the resultant structure is abnormal and disorganised. Experiments on teleost embryos at the pre-hatch stage have shown that similar disorganised structures cannot develop into adult fins (Birnie, 1934).

(c) Non-regenerative response to microsurgery

The results presented above indicate that distal portions of a fin fold are extremely sensitive to damage, whereas damage to central portions of the fin fold is soon repaired. These experiments provide evidence for a functional, as well as a spatial,

link between apical cleft cell morphology, extracellular matrix organisation and mesodermal cell migration. This is implied by the finding that after apical cleft cells and adjacent ectodermal cells have been removed, actinotrichia become misaligned and mesodermal cell migration is retarded.

It is interesting to note that epidermal cells positioned at the apex of damaged "balloon-like" fin folds do not form a single row of cleft-like cells. They do, however, form a series of disorganised surface invaginations. This finding indicates that positional information (Wolpert, 1969; 1971) which is restricted to the apex of a fin fold can still alter epidermal cell shaping in a fairly 'site specific' manner although normal cleft cell morphology can only be established after the set sequence of cell shape and extracellular matrix modulations described in Chapter 1 have been completed.

CHAPTER 4

THE IMPACT OF CYTOCHALASIN B ON FIN FOLD MORPHOGENESIS

INTRODUCTION

The previous chapters show that discrete changes in cell shaping are of crucial importance in generation of the early fin fold. These changes may be due to cytoskeletal modulation (Spooner, 1975; Clarke & Spudich, 1977; Gordon & Jacobson, 1978; Tucker, 1981) and/or changes in cell surface-contact and adhesion relationships.

The drug cytochalasin B has been shown to influence microfilament organisation in a number of systems (Pollard & Weihung, 1974; Stagno & Low, 1978; Spooner & Wessels, 1970). This Chapter describes a series of experiments in which developing fin folds were exposed to cytochalasin B to evaluate the role of actinoid fibres during fin fold morphogenesis.

RESULTS

Isolated tail buds were exposed to cytochalasin B (at 10μg/ml) for varying periods at a range of stages in fin fold morphogenesis. The results obtained are summarised in Fig. 5.

Fig. 5

This diagram shows the effect of exposing the developing fin fold to cytochalasin B for varying periods at different stages in morphogenesis. The following conventions are used:

S = Cell/Tissue shaping.
A = Actinotrichial alignment.
C = Cleft cell formation.
I = Intermediate filament organisation
Mt = Microtubule presence.

The vertical arrows indicate how each morphological feature develops during embryogenesis. Arrows shaded black indicate normal development, stippled arrows indicate abnormal development and white arrow indicate that a feature was not present. The hatched portions of parts A, B and C indicate the period of exposure to the drug. A time scale is provided at the left hand margin.

These results may be summarised as follows:

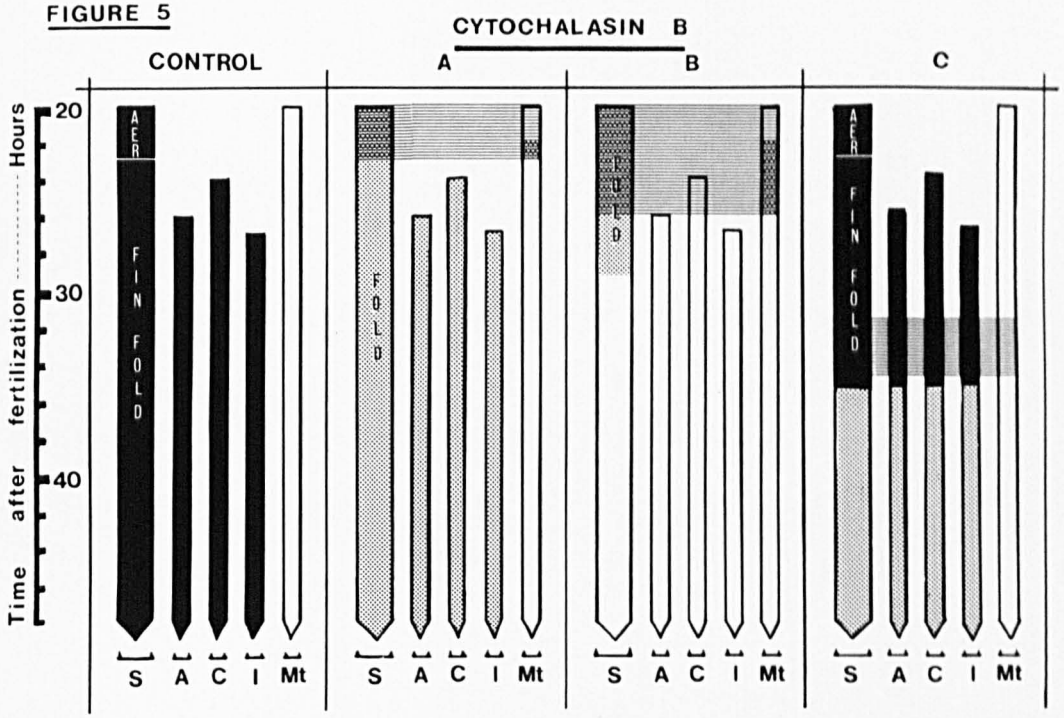
(CONTROL) Control cultures undergo normal morphogenesis.

(A) Exposure to cytochalasin B during apical ectodermal ridge development results in severe morphological abnormalities which are not repaired once the drug has been removed.

(B) Continued exposure to cytochalasin B during apical ectodermal ridge development and early fin fold morphogenesis leads to the construction of severely abnormal fin folds which disintegrate once the drug has been removed.

(C) Exposure to cytochalasin B during later phases of fin fold morphogenesis leads to the generation of morphological abnormalities once the drug has been removed.

FIGURE 5


CYTOCHALASIN B
CONTROL
A
B
C
Time after fertilization ---- Hours
20
30
40
AER
FIN FOLD
FOLD
S
A
C
I
Mt

(a) **The effect of cytochalasin B on apical ectodermal ridge development**

Tail buds which have been exposed to cytochalasin B during apical ectodermal ridge development do not form a normal apical ectodermal ridge (Fig. 5A). After 3 hours exposure to cytochalasin the putative fin fold epidermis forms disorganised clusters of irregularly shaped cells positioned along the anterior-posterior axis of the fin fold (Fig. 43). The remainder of the tail bud ectoderm i.e. all of the peridermis and all of the epidermis apart from that which forms cell clusters, sloughs away from the tail bud (Fig. 43).

When clusters of fin fold cells are left to recover from exposure to cytochalasin B, they develop into fin fold-like structures (Fig. 45). These 'folds' show some morphological abnormalities; cleft-like invaginations are found in epidermal cells which make up the side walls of a fold (Fig. 44) and actinotrichia and epidermal intermediate filaments are not aligned (Fig. 5A).

(b) **The effect of cytochalasin B on early fin fold development**

Cell clusters which are exposed to cytochalasin for a further three hours form abnormal fin fold-like structures (Fig. 5B). These folds form in the absence of flanking epidermis and overlying peridermis in the following way. Epidermal cells within a cluster start to separate from one another and form a central sub-epidermal space (Fig. 46). This space enlarges to form an extensive extra-cellular space which is bounded by a folded sheet of irregularly shaped epidermal cells (Fig. 47).

Epidermal cells within these folded structures do not undergo any of the shape changes described in Chapter 1. Thin sections indicate that these folds are ultrastructurally quite distinct from fin folds which develop in vivo. For example, they do not contain any actinotrichia, cross fibres, intermediate filaments, or cleft cells. However, epidermal cells within a fold do contain large numbers of microtubules which can not be observed in control cultures. Microtubules are mainly found close to cell membranes (Figs. 48 & 49) and in some cases they are included in small membrane bound bundles (Fig. 50). Centrosomes and associated microtubules (Fig. 51) were located close to the nucleus.

Abnormal folds do not recover from drug treatment. When they are placed in cytochalasin free culture medium they dissociate from the tail bud mesoderm within three hours (Fig. 5B).

(c) The effect of cytochalasin B on established fin folds

Established fin folds which have been left to develop normally are less sensitive to cytochalasin than putative fin fold epidermis. In established fin folds which have been exposed to cytochalasin for three hours morphological abnormalities only develop after the drug has been removed (Fig. 5C). Folds which are recovering from cytochalasin treatment have an enlarged sub-epidermal space (Fig. 52). Extracellular spaces develop between adjacent epidermal cells (Fig. 52) and cleft cells disintegrate (Fig. 53). Actinotrichia positioned near the base of a fold become disoriented (Fig. 54) although the proximo-distal orientation of apical portions

of these fibres is often preserved, even after cleft cells have disintegrated (Fig. 53).

(d) Control cultures. The effect of dimethyl sulphoxide (D.M.S.O.) on fin fold morphogenesis

Tail buds and associated fin folds which have been cultured for up to 48 hours in culture medium which contains 1% DMSO do not show any developmental abnormalities (Fig. 5. Control).

DISCUSSION

(a) The impact of cytochalasin treatment on cell shaping

Actinoid fibres have been shown to modulate cell and tissue shaping in a large number of developmental systems (for review see Wessels et al, 1971; Burgess & Schroder, 1979; Odell et al, 1981). In many of the systems studied these fibres form extensive arrays beneath the cell surface membrane. Contraction of these arrays in adjacent cells within a sheet causes the cells to become wedge-shaped which, in turn, leads to folding of the sheet of cells (Baker, 1965; Wessels & Evans, 1968; Wrenn & Wessels, 1969; Spooner, 1973). Co-ordination of cell contraction may be affected by the elastic properties of actin arrays themselves (Odell, et al, 1981).

The results presented above indicate that cell wedging plays a crucial role in generation the teleost apical ectodermal ridge.

Modification of the actin cytoskeleton prevents putative fin fold epidermal cells from becoming wedge-shaped and from forming a normal apical ectodermal ridge. Hence, it seems probable, that co-ordinated contraction of actin fibres which are located at the bases of epidermal cells, does generate the teleost apical ectodermal ridge *in vivo*. Such arrays are not observed in thin sections. This may be because they are less highly concentrated than those observed in other systems such as the developing salivary gland (Spooner & Wessels, 1970).

(b) Apical ectodermal ridge and fin fold construction during exposure to cytochalasin B

When putative fin fold epidermal cells are exposed to cytochalasin they do not undergo the normal sequence of cell shape changes seen in control cultures. However, they do clump together to form an elevated ridge-like structure which generates an unstable and disorganised fin fold. These fin folds develop in tail buds which have no peridermis and from which all of the epidermis apart from that which makes up the fold itself has been lost. This shows that the peridermis and flanking epidermis do not have a significant role in generation of the early fin fold and that the putative fin fold epidermal cells alone are responsible for generating the early fold.

Furthermore, these results also indicate that although actin based changes in cell shaping are extremely important in the initial phases of fin fold generation, other factors which are

confined solely to the putative fin fold epidermis are also important. For example, localised changes in cell adhesion and cell recognition may help to generate both the apical ectodermal ridge and early fin fold.

(c) Cell adhesion and the effect of cytochalasin on fin fold morphogenesis

When cytochalasin B is applied to tail buds which are undergoing the initial stages of fin fold morphogenesis, the putative fin fold epidermis is the only region of tail bud ectoderm which remains attached to the tail bud mesoderm. Both peridermis and flanking epidermis become detached. This indicates that the putative fin fold epidermis has specialised adhesion properties compared with the flanking epidermis and peridermis. Furthermore, these adhesion properties must be modulated during subsequent phases of fold morphogenesis as specific cells within the fin fold epidermis separate from one another and from mesoderm to generate the subepidermal space of the early fin fold. The role of cell adhesion during fin fold morphogenesis is discussed further in Chapter 5.

(d) The effect of cytochalasin B on extracellular matrix organisation within developing fin folds

Developing and established fin folds which have been exposed to cytochalasin invariably develop abnormalities in extracellular matrix secretion and/or organisation. A correlation between

extracellular matrix organisation and the cytoskeleton has been proposed by a number of investigators (Bernfield, Cohn & Banerjee, 1973; Bard & Hay, 1975; Hynes & Destree, 1978; Hay, 1982; Hay, 1983).

Fin folds become destabilised once the extracellular matrix has been disturbed by cytochalasin treatment. For example, disorganised folds, with no apparent extracellular matrix, assemble in the presence of cytochalasin and then dissociate from the tail bud soon after the drug has been removed. Similarly, established folds which have been exposed to the drug form "balloon-like" subepidermal spaces (similar to those described in Chapter 3) which also dissociate from the tail bud mesoderm soon after the drug has been removed.

The distal portions of actinotrichia which are contained in the cleft cell invaginations may play a special role in fin fold morphogenesis. For example, actinotrichia in the proximal portion of the subepidermal space become disoriented when folds are exposed to cytochalasin. In contrast, the distal curved portions of actinotrichia are more stable and maintain their anterior-posterior alignment during and after exposure to cytochalasin. This indicates that actinotrichial curving within the cleft cell invagination can increase the mechanical stability of these fibre arrays as suggested in Chapter 1.

(e) Cytochalasin B and the appearance of microtubules in epidermal cells

One of the most surprising observations described in this

chapter is that considerable numbers of microtubules are found in putative fin fold epidermal cells which have been exposed to cytochalasin. It is possible that these cytoskeletal components assemble in direct response to a change in the culture micro-environment produced by exposure to the drug-a similar situation may occur in hepatocytes -(Merland & Spiele, 1984). Alternatively very small numbers of microtubules may play a role in normal in vivo morphogenesis. Once morphogenesis is perturbed, however, turnover of microtubules may have been prevented and these cytoskeletal components might then accumulate in the cytoplasm until they appear in considerable numbers in thin sections.

CHAPTER 5

THE EFFECTS OF TUNICAMYCIN ON FIN FOLD MORPHOGENESIS

INTRODUCTION

The experiments described in Chapter 4 indicate that the actin cytoskeleton of fin fold epidermal cells probably plays a major role during fin fold morphogenesis. However, early fin morphogenesis is apparently not entirely actin dependent because some morphogenetic processes do occur in the presence of cytochalasin B. Such changes may have been mediated by modulation of cell-cell adhesion and/or extracellular matrix deployment within a developing fin fold.

The drug tunicamycin alters cell adhesion and extracellular matrix secretion by preventing glycosylation of glycoproteins (Tkacz & Lampen, 1975; Pratt, Yamada, Olden & Hascall, 1977; for review see Geisow, 1979).

This Chapter describes a series of experiments in which developing tail buds were exposed to tunicamycin in an attempt to assess the involvement of cell adhesion and extracellular matrix deployment during fin fold morphogenesis.

RESULTS

Tunicamycin (at 5 µg/ml) was applied to developing tail buds for varying periods at a range of different stages during morphogenesis.

The results which were obtained are summarised in Fig. 6. Control cultures of isolated tail buds in culture medium with 1% sterile distilled water added showed no developmental abnormalities (Fig. 6 control).

(a) **The effect of tunicamycin on early fin fold development**

Apical ectodermal ridges and fin folds do not form on tail buds which have been exposed to tunicamycin throughout the initial stages of fold morphogenesis (Fig. 6A). Instead, putative fin fold epidermal cells form an apical ectodermal ridge-like structure which consists of 6-9 epidermal cells/cross section grouped together above a subepidermal space which has an irregularly shaped cross-sectional profile (Fig. 55). This space contains a granular extracellular matrix which does not resemble any of the extracellular matrix components seen in control cultures.

As morphogenesis continues in the presence of tunicamycin (for up to 16 hours after initial exposure to the drug) the subepidermal space enlarges and some of the epidermal cells which surround the space form invaginations at their basal surfaces (Fig. 56). Although these invaginations are similar to those found in the cleft cells of control cultures, they do not contain the curved portions of actinotrichia found <u>in vivo</u>. Actinotrichia which are positioned within the proximal portion of a subepidermal space are not well oriented (Fig. 57).

When these abnormal ridge/fold-like structures are placed in tunicamycin-free culture medium they develop into abnormal

Fig. 6

This diagram shows the effect of exposing developing fin folds to tunicamycin for varying periods at different stages in morphogenesis. The following conventions are used:

S = Cell/tissue shaping.
A = Actinotrichial alignment.
C = Cleft cell formation.
E = Extracellular matrix deposition
Mt = Microtubule presence.

The vertical arrows indicate how each morphological feature develops during embryogenesis. Arrows shaded black indicate normal development, stippled arrows indicate abnormal development and white arrows indicate that the feature was not present. The hatched portions of parts A, B and C indicate the period of exposure to the drug. A time scale is provided at the left hand margin.

These results may be summarised as follows:

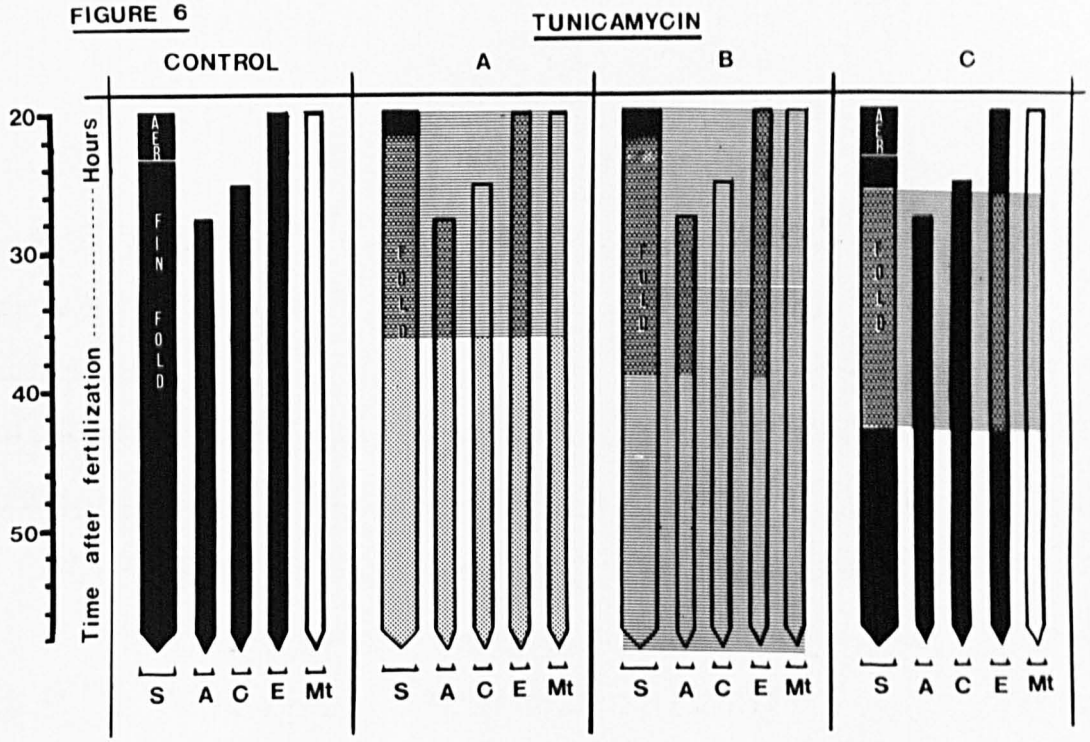
(CONTROL) Control cultures undergo normal morphogenesis.

(a) Exposure to tunicamycin throughout apical ectodermal ridge development and subsequent early fin fold morphogenesis leads to the construction of abnormal fin folds which do not recover after the drug has been removed.

(B) Exposure to tunicamycin for a longer period than that described in (A) leads to the disintegration of all fin fold-like structures.

(C) Exposure to tunicamycin after the fin fold has been established does lead to some changes in tissue shaping and extracellular deployment. However, these are "repaired" after the drug has been removed.

FIGURE 6


TUNICAMYCIN
CONTROL
A
B
C
20
30
40
50
Time after fertilization ---------- Hours
AER
FIN FOLD
S A C E Mt

fin folds (Figs. 6A,58). These folds contain an enlarged subepidermal space (Fig. 59) and are often capped distally by a row of cleft cells (Fig. 60). Surprisingly, these cleft cells contain large numbers of microtubules (Fig. 60); microtubules are not found in the cleft cells of folds that develop in control cultures. Actinotrichia situated within the proximal portion of a subepidermal space are not well oriented (Fig. 61) and do not align within the distal cleft cell invaginations. Granular extracellular matrix is distributed throughout the subepidermal space (Figs. 59, 60 & 61).

(b) The effect of tunicamycin on early and late fin fold morphogenesis

Fin folds are not present on tail buds which have been exposed to tunicamycin throughout the early and late phases of fold morphogenesis (up to 36 hours from apical ectodermal ridge formation) (Fig. 6B). The putative fin fold ectoderm of these tail buds forms a flat sheet over the tail bud mesoderm (Fig. 62). Abnormal fold-like structures like those that form on folds which have been exposed to tunicamycin for 16 hours are not observed.

(c) The effect of tunicamycin on the established fin fold

Established fin folds (5 hours after apical ectodermal ridge development) are less sensitive to tunicamycin that putative fin fold fold ectoderm (Fig. 6C). Established folds which have

been treated with tunicamycin for up to 16 hours develop some abnormalities similar to those described for folds which have been left to recover from a 16 hour exposure to the drug during the initial phases of morphogenesis. They contain enlarged subepidermal spaces (Fig. 63) which are packed with granular extracellular matrix (Fig. 64). Cleft cell organisation and actinotrichial orientation are not affected by the drug (Fig. 64). Soon after rinsing in sterile culture medium to remove the drug, the fin fold undergoes the normal sequence of cell and tissue shape changes similar to those observed in control cultures (Fig. 6C).

DISCUSSION

(a) The role of cell adhesion and extracellular matrix deployment in early fin fold morphogenesis

Changes in cell adhesivity and extracellular matrix secretion may play an important role in the morphogenesis of a number of developmental systems. For example, the shape of various epithelial cells is thought to be determined by specific changes in extracellular matrix secretion (Gospodarwicz, Greenburg & Birdwell, 1978; for review see Saxen, Ekblom & Thesleff, 1980; Hay, 1982). Furthermore, changes in cell adhesivity have been show to alter the morphogenesis of the ciliary body (Bard & Ross, 1982) and arthropod leg segment (Mittenthal & Mazo, 1983) as well as other tissues (for review see Phillips et al, 1977).

The results presented in this chapter show that when putative fin fold epidermis is exposed to tunicamycin (a drug which modulates

cell adhesivity and extracellular matrix secretion (see review by Geisow, 1979)), epidermal cells do not undergo the normal sequence of shape changes recorded for control cultures. This indicates that cell adhesion and/or extracellular matrix deployment play an important role in establishing the apical ectodermal ridge and early fin fold.

A complex series of modulations of cell contact relationships within the tail bud epidermis may participate in early fin fold morphogenesis in the following way.

Initially, the majority of putative fin fold epidermal cells may become highly adherent to each other. This would cause cells to maximise surface contact by clumping together to form a ridge along the length of a tail bud. On the other hand, putative fin fold epidermis at the base of a ridge might have specialised contact relationships which would cause it to become adherent to fin fold ectoderm, flanking ectoderm and also to mesoderm. In this way it could provide a spatial link between the apical ectodermal ridge and the remainder of the tail bud. Once the apical ectodermal ridge has been formed, highly localised changes in epidermal cell contact along the centre of the ridge may generate the sub-epidermal space and convert the ridge into an early fin fold.

A comparison of the results presented in this chapter, with those presented in Chapter 4, indicates that cell adhesivity and/or extracellular matrix secretion are mainly involved in modulating the spatial relationships between different epidermal cells within a developing fold. In contrast, cytoskeletal deployment probably

plays an important role in modulating the specific shapes of individual cells within a fold. There is substantial evidence that intracellular and extracellular control of morphogenesis is co-ordinated by transmembrane links between the cytoskeleton and extracellular matrix. (See review by Hay, 1983).

(b) The role of cell adhesion and extracellular matrix deployment in stabilising the established fin fold

Cell adhesion and extracellular matrix deployment are thought to play an important role in the stabilisation of vertebrate tissue. For example, developing salivary gland epithelium is thought to be supported by extracellular matrix material which is secreted between the epithelium and underlying mesoderm (Spooner, 1973; for review see Saxen, Ekblom & Thesleff, 1980).

In this context it is interesting to note that extracellular matrix deployment may play an important role in fin fold stabilisation. Fin folds which develop abnormalities after exposure to tunicamycin do not contain the extracellular cross fibres described in Chapter 1. This finding corresponds with the suggestion made in Chapter 1 that cross fibres may play an important role in holding the side walls of the fold together before large scale polymerisation of actinotrichia occurs.

(c) Cleft cell morphogenesis and the effect of tunicamycin

Experiments in which tunicamycin is applied to developing tail buds show that extracellular matrix secretion and/or modulation

of cell adhesivity influence the development of the cleft cell invagination. In this respect development of the cleft cell invagination is similar to a number of embryogenetic processes in which a lumen is formed as a result of cell separation and changes in cell contact relationships; for example, mammary gland development(Hogg, Harrison & Tickle, 1983) and parotid gland development (Redman & Sreebny, 1971). In these systems however, co-ordinated changes in the adhesivity of a large number of different cells generates extracellular spaces. It is surprising that in the developing fin fold such changes can be confined to a small region of a cleft cell membrane.

It is interesting to note that sensitivity to tunicamycin is lost once cleft cell invaginations are established and they contain normal arrays of extracellular fibres. This indicates that extracellular matrix fibres may stabilise the cleft cell invagination as well as the remainder of the subepidermal space as suggested in Chapters 1 and 4.

(d) The formation of microtubules in fin fold epidermis which has been exposed to tunicamycin

In Chapter 4 it is reported that microtubules are found in fin fold epidermis which has been exposed to cytochalasin. Surprisingly, large numbers of microtubules also assemble in the cleft cells of fin folds which have been exposed to tunicamycin This observation again raises the question of microtubule involvement during fin fold morphogenesis. Are small numbers of microtubules

involved in fold morphogenesis, or do microtubules assemble as some peculiar response of fin fold epidermis to drug treatment? This issue is investigated in the following chapter.

CHAPTER 6

THE EFFECT OF NOCODAZOLE ON FIN FOLD MORPHOGENESIS

INTRODUCTION

The appearance of microtubules in the epidermis of developing fin folds which have been exposed to cytochalasin B and tunicamycin raises important questions. Do microtubules actually have some functional significance during normal morphogenesis? Do they occur with very low spatio-temporal frequency so that they have escaped detection unless this frequency is altered by drug application? Alternatively, are 'drug induced' microtubules non-functional and formed only as a response to drug exposure?

The drug nocodazole has been shown to induce the depolymerisation of microtubules (De Brabander et al, 1975; Hoebeke, Van Nigen & De Brabander, 1976). It is rapidly effective at very low concentrations and may be eluted from tissue samples more easily than many of the other microtubule depolymerising agents (De Brabander et al, 1975; Engelborghs & Lambier, 1980). Nocodazole was applied to fin folds at several stages in morphogenesis to explore the possibility that fin fold morphogenesis might be perturbed in some way.

RESULTS

Nocodazole was dissolved in 1% DMSO and incorporated in the culture medium at up to 3x the effective dose used by other workers (3 µg/ml) (De Brabander & De Mey, 1980; De Brabander et al, 1981). Control cultures of tail buds in culture medium which contained 1% DMSO showed no developmental abnormalities (Fig. 7 control).

(a) Exposure to nocodazole during apical ectodermal ridge and early fin fold morphogenesis

The development of fin folds is not sensitive to nocodazole for a 6 hour period between 20 and 26 hours post fertilisation. Fin Folds which develop on tail buds exposed to nocodazole throughout this period show no morphological abnormalities during or after exposure to the drug (compare Figs, 7 control & 7A).

(b) Exposure to nocodazole until the stage of fin fold expansion

The fin fold starts to expand approximately 10 hours after apical ectodermal ridge development (see Chapter 1). Fin folds which are exposed to nocodazole throughout apical ectodermal ridge development and beyond the point at which fin fold expansion ordinarily occurs in culture, do show some developmental abnormalities (Fig. 7B) These folds do not expand but instead become distorted in cross sectional profile (Fig. 65) and the arrangment of actinotrichia near the base of such folds develops abnormalities (Fig. 66).

Fig. 7

Diagram showing the effect of exposing the developing fin fold to nocodazole for varying periods at different stages in morphogenesis. The following conventions are used:

S = Cell/tissue shaping
A = Actinotrichial alignment.
C = Cleft cell formation.
I = Intermediate filament organisation.

The vertical arrows indicate how each morphological feature develops during embryogenesis. Arrows shaded black indicate normal development, stippled arrows indicate abnormal development and white arrows indicate that a feature was not present. The hatched portions of parts A, B and C indicate the period of exposure to the drug. A time scale is provided at the left hand margin.

These results may be summarised as follows:

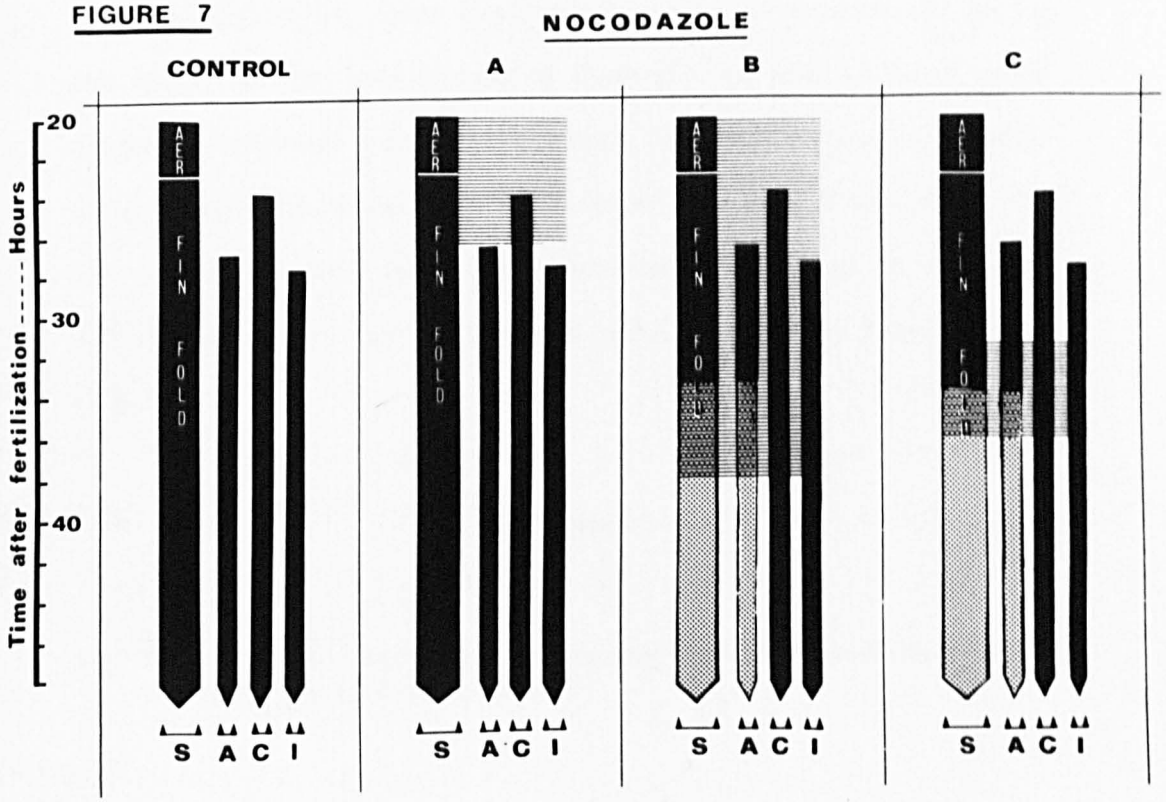
(CONTROL) Control cultures undergo normal morphogenesis.

(A) Exposure to nocodazole during apical ectodermal ridge and early fin fold development does not alter morphogenesis.

(b) Exposure to nocodazole during fin fold construction up to the phase of fin fold expansion results in some abnormalities in tissue shaping and actinotrichial organisation. These abnormalities are retained after the drug has been removed.

(C) Exposure to nocodazole during part of the phase of fin fold expansion leads to similar developmental abnormalities to those described in (B) above.

FIGURE 7


NOCODAZOLE
CONTROL
A
B
C
Time after fertilization -----Hours
20
30
40
AER
FIN FOLD
S A C I

(c) Exposure to nocodazole during the stage of fin fold expansion

Established fin folds which are exposed to nocodazole during the phase of fin fold expansion (between 10 and 15 hours after apical ectodermal ridge generation) (Fig. 7C) develop similar abnormalities to those described above (compare Figs. 7B & 7C) for fin folds which have been continuously exposed to the drug for 18 hours from the start of the apical ectodermal ridge generation.

DISCUSSION

(a) The role of microtubules during apical ectodermal ridge and early fin fold development

High concentrations of nocodazole, (up to 3x those used by previous workers (De Brabander et al, 1981), do not significantly alter cell or tissue morphology during the early stages of fin fold development. However, the block of expansion which is observed when nocodazole is applied to expanding fin folds, indicates that the drug does penetrate the developing tail bud. It is probable that the microtubule arrays described in Chapters 4 and 5 do not play a significant functional role in the early phases of fin fold morphogenesis and may therefore be induced by exposure to cytotoxic drugs. It has been reported that microtubule polymerisation may be induced in other experimental systems in response to changes in the composition of the culture

medium, for example, microtubules assemble when detergents are introduced into solutions of isolated rat brain tubulin (Daleo, Piras & Piras, 1977) and cultures of hepatocytic cells (Merland & Spiele, 1984). It is not clear why microtubules form in this idiosyncratic way in specific cell types. However, this response may be worth considering in future assays of drug activity.

(b) **The role of microtubules during fin fold expansion**

Microtubules do appear to play an important role during fin fold expansion. It has been suggested that the fin fold expands as a result of localised cell division within the fin fold epidermis and/or recruitment of flanking epidermal cells into the fold (Chapter 1; Dane & Tucker, 1985).

Nocodazole prevents mitosis (De Brabander et al, 1975) and therefore probably blocks localised cell division within a fold. If this is the case then the changes in fold shaping and actinotrichial alignment induced by nocodazole treatment may be a consequence of actinotrichial polymerisation within the restricted volume of a fold which can not expand.

CHAPTER 7

SUPRACELLULAR MICROTUBULE ALIGNMENTS ASSOCIATED WITH THE PATTERN OF EXTRACELLULAR MATRIX DEPOSITION IN CERTAIN FISH SCALES

INTRODUCTION

The previous chapters deal with the functional correlation between cytoskeletal organisation and extracellular matrix deployment during a certain instance of tissue shaping in Brachydanio. This chapter deals with an immunofluorescence and transmission electron microscopical assesssment of the spatial correlation between the pattern of extracellular matrix deposition and cytoskeletal orientation in another tissue in Brachydanio and certain other teleosts, namely the adult body scale.

RESULTS

(a) General organisation of scales and associated cell layers in Brachydanio, Hyphessobrycon and Salmo

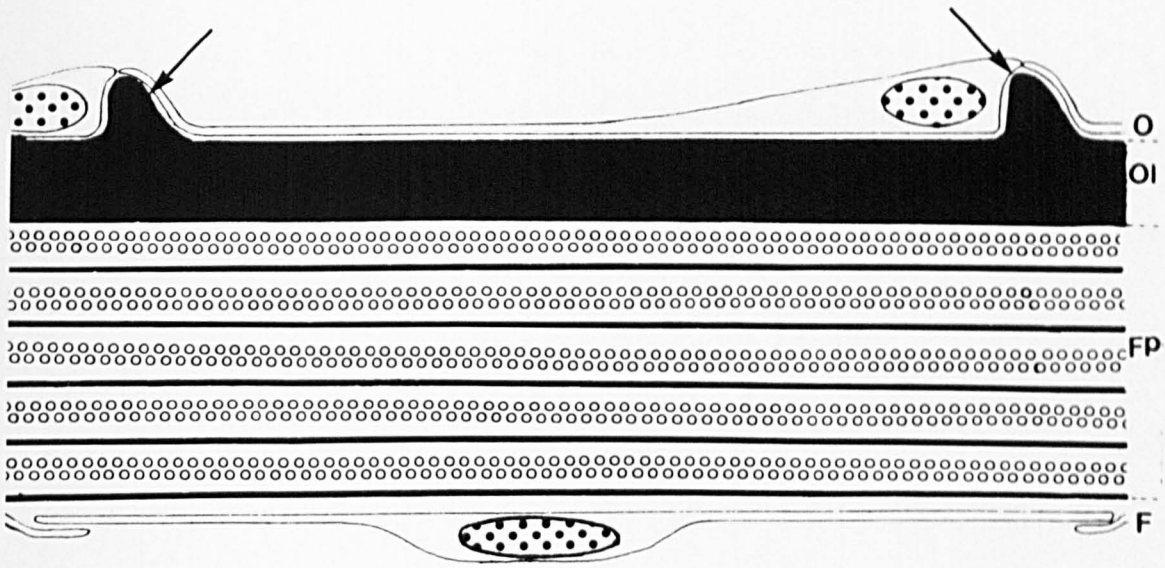
The general organisation of the teleost scales studied is summarised in Fig. 8 and described here as a prelude for understanding microtubule distribution in relation to cell positioning and scale architecture.

The teleost scale is composed of a bony plate of extracellular material which is surrounded by a layer of flattened fibroblastic cells. The bony plate is divided into an external layer or ridged and grooved osseous material (osseous matrix) which is underlain

Fig. 8

Schematic diagram showing the shapes and arrangements of osteoblasts (O) and fibroblasts (F) which cover a scales osseous layer (OL) and fibrillary plate (FP) respectively. This diagram is based on sections cut near the anterior of Brachydanio scales and at right angles to the plane of each scale and the longitudinal axes of their circuli. The positions of the scarp-shaped cross-sectional profiles of the osseous circuli are indicated by arrows. Nuclei are stippled. The number of layers of collagen fibres represented in the fibrillary plate is approximately 70% of that found in the scales examined.

FIGURE 8



10 µm

Fig. 9

Schematic diagram showing the arrangement of circuli (C) and radii (R) on the outer surface of the osseous layer of a Brachydanio scale. The anterior edge of the scale is oriented towards the top of the figure.

FIGURE 9

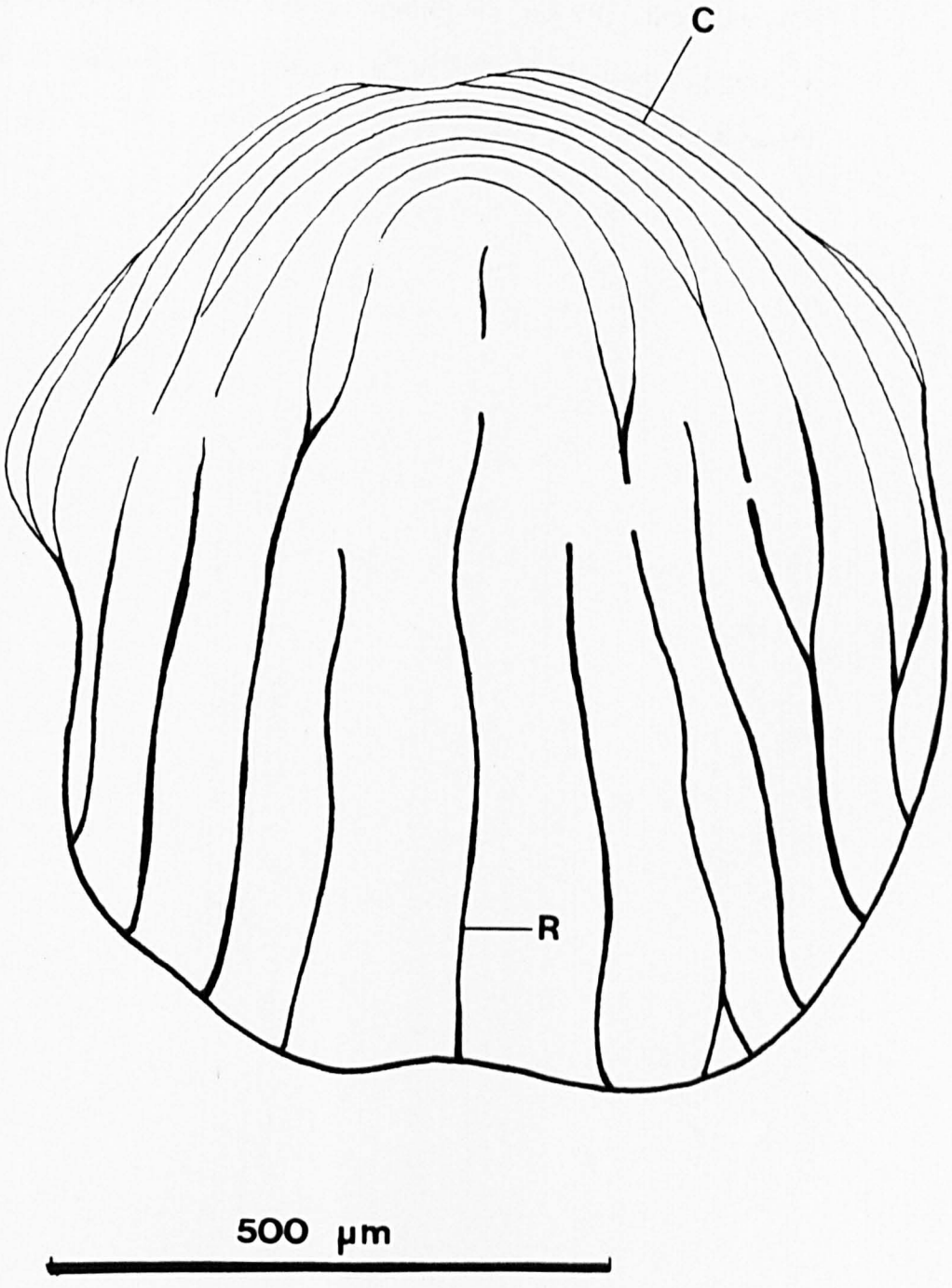


Fig. 10

Schematic diagram showing the arrangement of circuli (C) and radii (R) on the outer surface of the osseous layer of a Hyphessobrycon scale. The anterior edge of the scale is oriented towards the left hand margin of the figure.

FIGURE 10

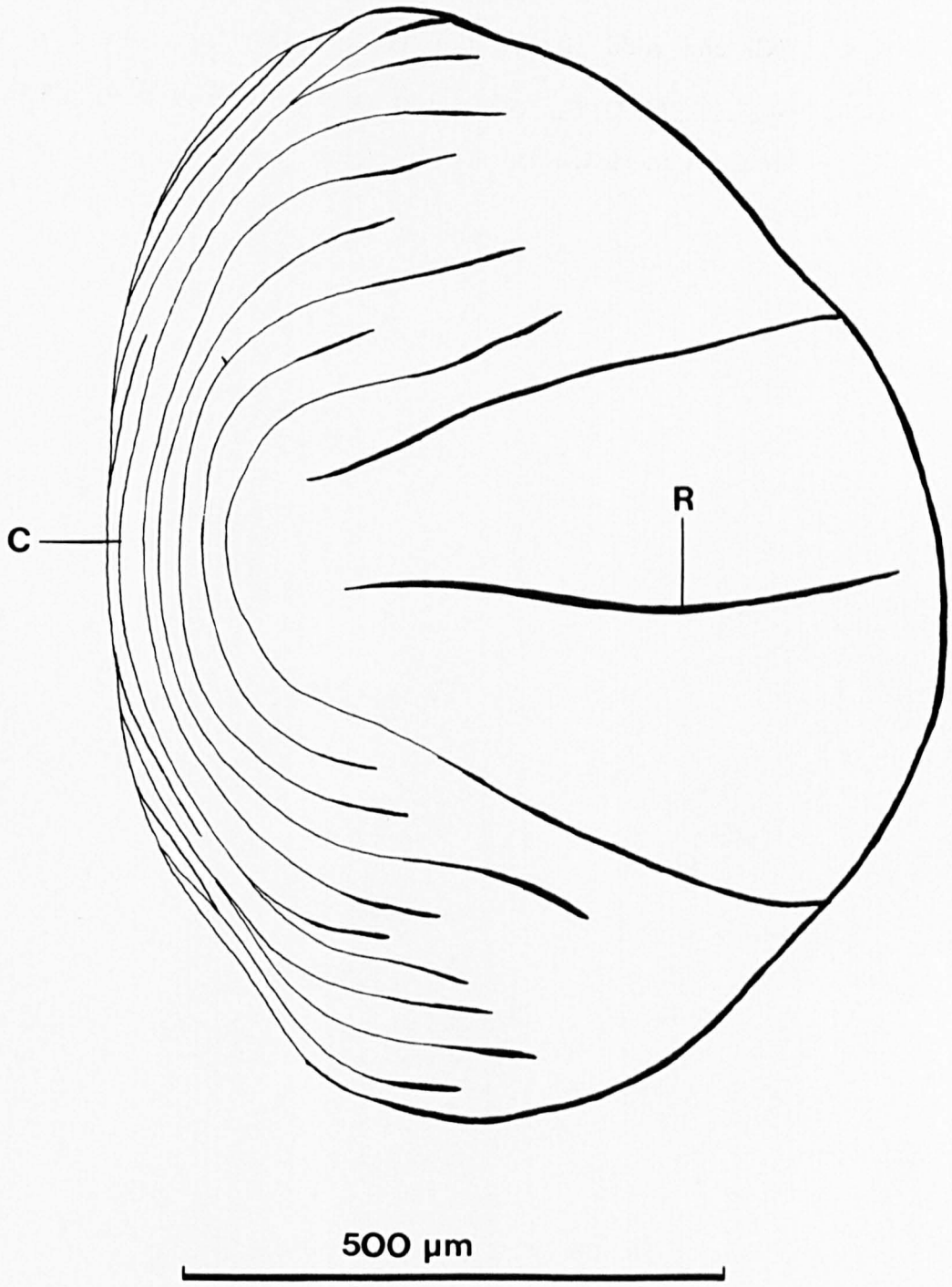
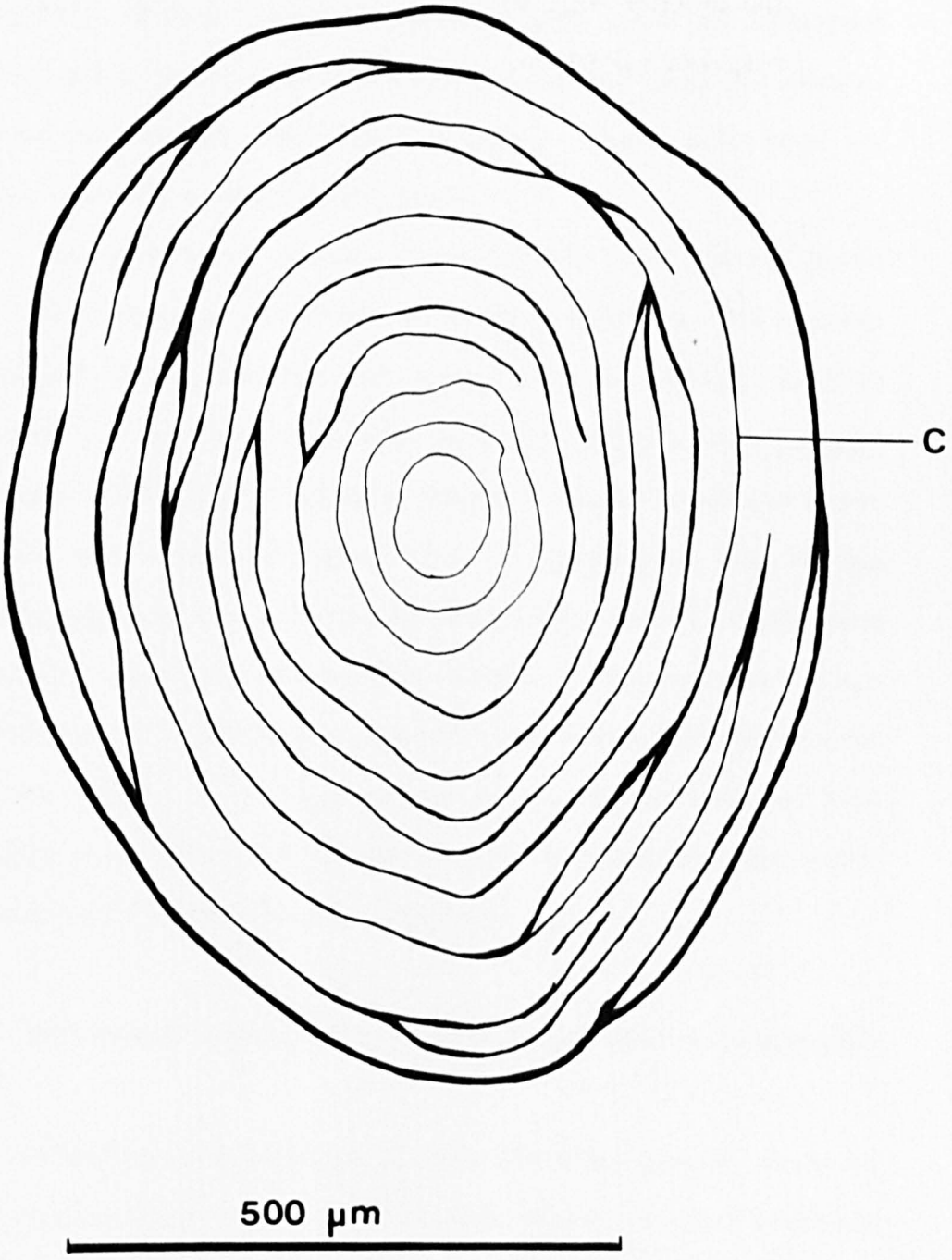


Fig. 11

Schematic diagram showing the arrangement of circuli (C) on the outer surface of the osseous layer of a Salmo scale. The anterior edge of the scale is oriented towards the left hand margin of the figure.

FIGURE 11



by an internal layer of orthogonally arranged collagen fibres (fibrillary plate) (Fig. 68). Cells which cover the osseous layer are referred to as osteoblasts, whilst those which cover the internal surface of the fibrillary plate will be referred to as fibroblasts (Fig. 8). Several layers of epidermal cells cover the osteoblast layer and a layer of dermal cells and pigment cells are situated inside and against the fibroblast layer. These cells have not been examined in detail in this study.

The pattern of osseous ridges (circuli) and grooves (radii) of the external osseous matrix of scales is highly variable between different teleost families and orders as is the overall shape of scales. In the cyprinids studies here (Brachydanio and Hyphessobrycon) scales are irregularly shaped (ctenoid) with concentric circuli and radially arranged radii. In Brachydanio (Fig. 9) and Hyphessobrycon (Fig. 10) circuli are closely spaced (approximate separation 10μm) at the anterior margin of the scale and more widely spaced (approximate separation 40μm) towards the posterior of the scale. In contrast, scales of the salmonid studied here (Salmo trutta fario) are roughly circular (cycloid) with concentric osseous ridges or growth annuli (Fig. 11).

(b) Microtubule alignment in the osteoblast layer of Brachydanio

Osteoblasts are arranged in rows which are parellel to circuli in regions where each 'intercirculus space' is one osteoblast wide. The anterior and posterior margins of adjacent osteoblasts run alongside each other close to the crest of the ridge-like

circuli (Figs. 8 & 67). Each osteoblast is much thicker (up to 4μm) at its anterior edge, where it flanks the posterior side of a circulus, than it is more posteriorly. The thick anterior portion of an osteoblast contains its nucleus (Figs. 8 & 67) and juxtanuclear centrosome. Each osteoblast flattens out posteriorly into an extremely thin (about 0.2μm thick) lamella-like cell portion (Fig. 67).

Most of the microtubules close to the anterior margins of these osteoblasts are aligned with and run parallel to the steep posterior flank of the adjacent circulus (Fig. 67). In immunofluorescence preparations the decorated microtubules at the anterior margins of the osteoblasts contribute to fluorescent bands and strands which coincide spatially with the positions and alignments of circuli (Fig. 69 arrows). However, in more posterior portions of these cells, most of the microtubules are oriented approximately at right angles to circuli and some of them span 'inter-circulus' spacings (Fig. 69). Examination of thin sections reveal microtubules concentrated along the anterior margins of these osteoblasts (Fig. 67). However, profiles of microtubules were rarely encountered in the more posterior lamelliform portions of the cells where immunofluorescence microscopy demonstrates the presence of aligned microtubule portions with an average spacing of 2μm, which were apparently not preserved during preparation for electron microscopy.

Microtubule arrangement varies in ways that are correlated with the spacing of circuli and whether osteoblasts are located in regions where circuli or radii occur. For example, where circuli are closely spaced (about 15-20μm apart) microtubules

show marked alignment at right angles to circuli (Fig. 69). However, where circuli are spaced further apart (about 50μm) microtubules are less precisely aligned with respect to each other and occur at a wider range of angles with respect to circuli (Fig. 70). In contrast, where circuli and radii approach each other, circuli are discontinuous and most widely spaced (about 60μm apart) microtubule arrangment only approximates to the orderly patterns described for regions where circuli are closest together (compare Figs. 69 & 71). In the central and posterior portions of scales, radii occur and there are no circuli. Adjacent radii are separated by up to 150μm which is several times greater than the major dimensions of individual osteoblasts. Microtubules in these regions do not exhibit any well defined patterns of alignment and, in general, most microtubules radiate out from the central nucleus containing portions of the cells (Fig. 72). Strips of weak fluorescence which coincide with the location of radii are observed in scales which have been exposed to FITC conjugated antibody (Fig. 72 arrows). Control preparations indicate that this is apparently due to non specific binding, or accumulation of the secondary antibody in the grooved radii or on components that run closely alongside them.

(c) Microtubule alignment in the fibroblast layer of Brachydanio

Microtubules are radially arranged with respect to a fairly central site in each fibroblast. Most of these microtubules curve into alignment with each other, and those in neighbouring cells at a distance of around 7μm from the central site (Fig. 73).

The aligned portions of microtubules have a length of around 35μm. Examination of thin sections reveals that centrosomes are situated near the centres of cells close to their nuclei, and probably occupy the central sites described above. The central nucleus-containing regions of the fibroblasts have thicknesses of up to 1.5μm, whereas the peripheral regions which contain most of the aligned microtubules are extremely thin and lamelliform with thicknesses of about 0.2μm (Fig. 74). As in the osteoblasts, very few microtubule profiles were encountered in lamelliform portions of fibroblasts.

In some regions of scales the aligned portions of microtubules traced out a set of arc-like alignments across the fibroblast layer (Fig. 73). In others they were oriented straight across the layer. Phase contrast and differential interference contrast examination of fixed and unfixed scales show that the alignment of collagen fibres in the fibrillary plate exhibits a similar pattern of 'arcs' and 'straights'. However, using these techniques, it was not possible to differentiate between collagen orientation at the layer closest to the upper secretory surfaces of fibroblasts and collagen orientation at other levels in the fibrillary plate. Hence, direct demonstration of co-alignment of microtubules and collagen fibres at the level in question was not achieved.

(d) Microtubule alignment in the scales of Hyphessobrycon

Hyphessobrycon innesi and Brachydanio rerio are both members of the order Cypriniformes. In addition, they both have ctenoid

scales with similar patterns of circuli and radii (compare Figs. 9 & 10). Correlated with this is the finding that microtubule arrangment and alignment in the osteoblast and fibroblast layers of scales in these two species are also very similar. For example, in both species microtubule portions are most exactly aligned, and oriented most precisely at right angles to circuli, in regions where circuli are most closely spaced (compare Figs. 69, 70, & 71 with 75 & 76). Furthermore, in some Hyphessobrycon osteoblasts which are associated with circuli it is evident that microtubules apparently curve fairly abruptly out of alignment with circuli at a number of points along the anterior margin of the cell (Fig. 79). The portions of microtubules oriented at right angles to circuli are therefore likely to be the distal portions of the microtubules which are aligned parallel to circuli and have proximal portions close to the anteriorly situated juxtanuclear centrosomes.

Many osteoblasts in the central portions of scales, where radii occur, detached from scales during preparation for immunofluorescence microscopy. In those that remained, the radial arrangment of microtubules was usually more pronounced and orderly that it was in the radius-associated osteoblasts of Brachydanio (compare Fig. 72 with Figs 77 & 78). In addition, marginal groupings of microtubules which appeared to be the distal portions of the radially arranged microtubules extended around the edges of flattened Hyphessobrycon osteoblasts (Figs. 77 & 78).

Microtubule arrangment in Hyphessobrycon fibroblasts was strikingly similar to that described for fibroblasts in Brachydanio scales (Fig. 80).

(e) **Microtubule arrangement in the scales of salmo**

The surface topography of the osseous layer of the scales of Salmo (order isospondyli) is distinctly different from that of the cypriniform species considered above (compare Figs. 9, 10 & 11). The immunofluorescence procedure does not clearly reveal microtubule distribution in the fibroblast layers of salmo scales and microtubules are not as readily detectable in the osteoblast layers as they are in those of the two Cypriniform species (Fig. 81). Furthermore, microtubules do not exhibit well defined patterns of alignment with respect to adjacent circuli even in regions where these are as closely juxtaposed as circuli in Brachydanio and Hyphessobrycon (compare Figs. 70, 76 & 81).

(f) **Influence of preparative procedures for immunofluorescence and electron microscopy**

The fluorescent images of microtubules in the fibroblasts and osteoblasts described above, are probably obtained from portions of scales where only the osteoblast and/or fibroblast cell layers remained attached to the scales. Evidence for this is obtained from light microscopial examination of methylene blue stained 1μm thick sections of undecalcified Brachydanio scales and associated cells which have been removed from fishes in the same way as scales prepared for immunofluorescence studies (see Materials and Methods). In many regions of most scales, the epidermal and dermal cells slough away and only some regions of the fibroblast and osteoblast cell layers shown in Fig. 8 remain attached.

However, several layers of epithelial cells also remain attached to the osteoblast layer of each scale for distances of up to 300μm from the posterior margin of a scale. These epithelial layers exhibit a fairly pronounced and diffuse fluorescence, after the immunofluorescence procedure, which is sufficiently substantial to mask fluorescent images of microtubules which might otherwise be discriminated in cells associated with the most posterior portions of scales.

Thin sectioning of undecalcified scales and associated cells was attempted. Deformation and tearing of these sections is so extensive that most details of cytoplasmic organisation are obscured. These difficulties are not encountered after decalcification (see Materials and Methods). However, there is evidence that the decalcification procedure leads to substantial digestion or breakdown of microtubules in lamelliform portions of scale-associated cells (see above).

DISCUSSION

(a) Immunofluourescence microscopy and decoration of microtubules in cells associated with teleost scales

Microtubule layout can be examined fairly readily using immunofluorescence microscopy in the osteoblast and fibroblast layers associated with the secretion of ctenoid scales of at least four teleost species. Namely a bericyform species represented by the squirrel fish (Byers, Fujiwara & Porter, 1980) and three cypriniform species including the goldfish (Cyprinidae) (Byers, Fujiwara & Porter, 1980), the zebra fish (Cyprinidae) and the

neon tetra (Characidae). This approach was less successful in attempts to monitor microtubule distribution in cells associated with the cycloid scales of a trout (Salmo trutta fario, order Isoponyli).

There is a possibility that microtubule alignment is a transient phenomenom in the cycloid scale and only associated with the initial establishment of circuli. Alternatively, microtubule alignment and orientation could be perturbed during preparation of salmonid scales for microscopy. If this is the case then the technique will probably work well with most cypriniform species, but the question of whether it is widely applicable in its present from to members of the other teleost orders remains uncertain.

(b) Microtubule alignment and supracellular patterns of extracellular matrix in fish scale fibroblasts

Microtubule alignment in the fibroblast layer of Brachydanio and Hyphessobrycon scales is very similar to that described in goldfish scale fibroblasts. In the goldfish scale, microtubule alignment is strikingly correlated with the alignment of fibres in the collagen layer of the fibrillary plate which is closest to the secretory surfaces of the fibroblasts (Byers, Fujiwara & Porter, 1980).

This may also be the case for Brachydanio and Hyphessobrycon where there is also a close similarity between supracellular patterns of microtubule alignment in fibroblasts and fibre alignment in certain collagen layers of the fibrillary plate.

Direct demonstration of microtubule/collagen fibre coalignment is achieved in the goldfish by comparing phase contrast images of fibres in collagen layers next to the secretory surfaces of fibroblasts with immunofluorescent images of microtubules in the same fibroblasts. This was not accomplished in Brachydanio and Hyphessobrycon because it is not possible to detect the orientation of the collagen fibres closest to the fibroblasts using phase or differential interference contrast microscopy. Unfortunately transmission electron microscopy does not clarify this issue because most of the aligned portions of fibroblasts are not preserved. However, coalignment of Brachydanio fibroblast microtubules and collagen fibres closest to the fibroblast is apparent in a micrograph published by Waterman (1980, Fig. 11).

(c) Microtubular alignment and supracellular patterns of extracellular matrix in fish scale osteoblasts

Microtubule alignments in osteoblasts are strikingly correlated with the arrangement of osseous circuli on Brachydanio and Hyphessobrycon scales. What is the functional significance of these microtubule alignments? There is an intriguing possibility that the groupings of microtubules which run closely alongside circuli form part of the mechanism which specifies the orientation of circuli and determines where these ridge-shaped thickenings will be secreted onto the outer surface of the osseous layers. Microtubule portions which run at right angles to circuli often

span the inter-circulus spaces. Are these microtubules used as an intra-cellular mechanism to measure out inter-circulus spacings? This possibility is especially pertinent to investigations where the spacings and widths of osseous ridges have been used to estimate the ages and growth rates of their owners (for example Tesch, 1955; Galkin, 1958; Larrañeta, 1964; Ricker, 1968).

An alternative possibility is that the orderly shaping and positioning of osteoblasts in the vicinity of circuli and the alignments of microtubules in osteoblasts and fibroblasts are features that occur as a response to, rather than being the cause of, the orderly patterns of extracellular matrix material which they secrete. For example, the oriented responses of fibroblasts in 3-dimensional collagen substrates is well established (see Löfberg & Ebendal, 1980).

(d) Centrosomal microtubule-organising centres and the control of microtubule alignment

Investigation of several animal tissue cell types has shown that many of their microtubules grow out from their centrosomes (Brinkley, Fuller & Highfield, 1975; Osborn & Weber, 1976(a); Gould & Borisy, 1977; Brinkley et al, 1981; for reviews see McIntosh, 1983; Bornens & Karsenti 1984). Presumably this is also the case for microtubules which project from the vicinities of centrosomes in the cells examined in this study. Some of these cells provide examples of three types of situations where the distal portions of microtubules bend into orientations which are markedly different from those of their proximal portions (Fig. 12). For

example, in flattened Hyphessobrycon osteoblasts which are positioned near the middles of scales, microtubules are more or less radially oriented with respect to cell centres and bend into alignment with each other to form a 'marginal band' at the edges of the cell (Fig. 12A). Such bending may be a response to interactions which are induced by encounters between microtubules and cell edges similar to those described by Dentler, Pratt & Stephens, (1980) and Tucker (1981). The tendency of microtubules which radiate from the centres of flattened cells to extend around the edges of such cells has been observed by others (Osborn & Weber, 1976(b); Weber & Osborn, 1979; Brinkley et al, 1980; Byers, Fujiwara & Porter, 1980). Conversely, in osteoblasts which are located between closely spaced circuli at the anterior of the scale, microtubules bend away from their alignments alongside the cell edges into another alignment (Fig. 12B). These microtubules, or small groups of microtubules, bend away from the main anterior bundle at a series of regularly spaced loci. Evidently conditions which promote re-alignment of certain microtubules at these loci do not modify the orientation of the remainder. In contrast, fibroblasts contain radially oriented microtubules which bend into alignment with each other at localities which are not associated with cell edges (Fig. 12C).

The changes in microtubule orientation and alignment considered above are situated at considerable distances (up to 30μm) from centrosomes (Fig. 12B). It is far from clear how such re-orientations are accomplished. Bends may initially develop at the locations where curving portions of microtubules have

Fig. 12

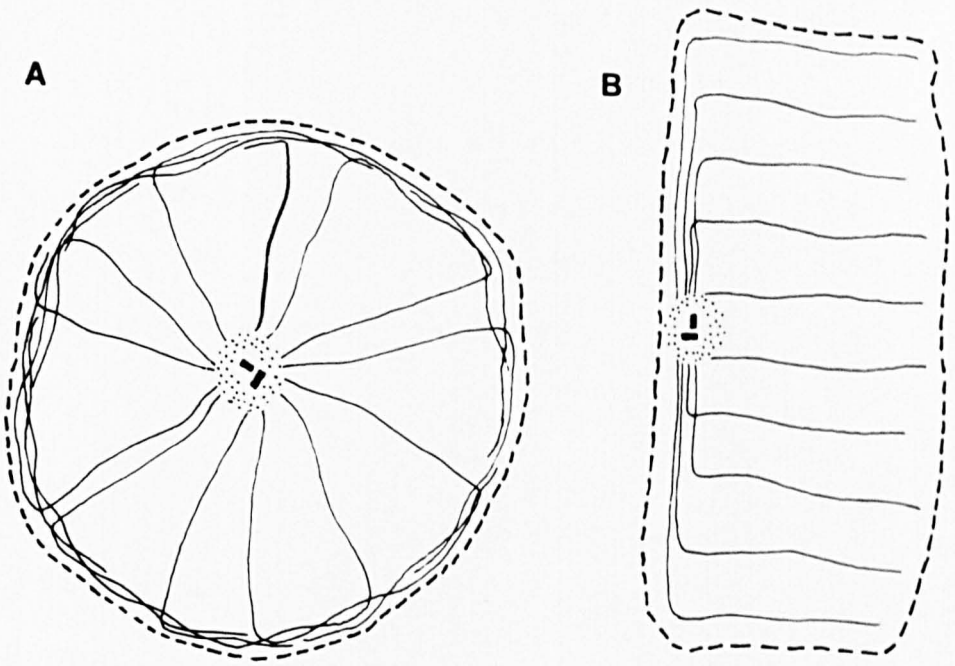
Schematic diagram showing the arrangement of microtubules with respect to the margins of cells (broken lines) and the apparent locations of the proximal ends of microtubules with respect to centrosomes in the scale associated cells of certain teleosts. Centrosomes are represented by a pair of black rectangles (centrioles) surrounded by stippling (pericentriolar material).

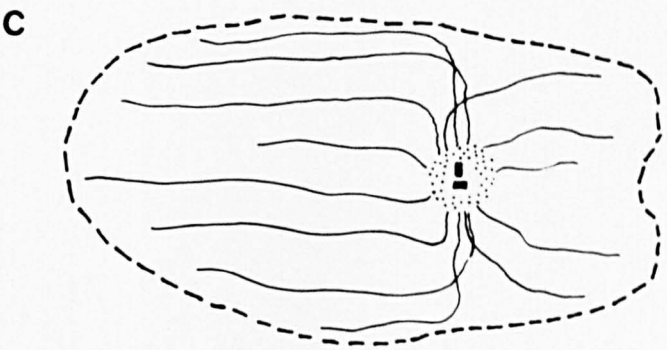
A. An osteoblast near the central portion of a Hyphessobrycon scale.

B. An osteoblast near the anterior edge of a Hyphessobrycon or Brachydanio scale where circuli are most closely spaced.

C. A fibroblast of a Hyphessobrycon or Brachydanio scale.

FIGURE 12





50 µm

been observed. If this is the case, then such venues are presumably too remote from the centrosomes for these microtubule organising centres to make any direct contribution to the events which reorganise microtubule alignment. These observations are complemented by studies of certain other cell types where microtubules seem to detach from centrosomes and migrate away from them as microtubule positioning proceeds (Warren, 1974; Tucker, 1981; Vorobjev & Chentsov, 1983). In all of these cases there is a considerable degree of decentralised control of microtubule organisation which apparently occurs in addition to the initial establishment of microtubular specifications (such as those for control of microtubule number and polarity) which may be provided by the centrosomal microtubule organising centres (for reviews see McIntosh, 1983; Bornens & Karsenti, 1984; Tucker, 1984).

CONCLUSION

1. FIN FOLD MORPHOGENESIS AND FISH SCALE MORPHOLOGY - A RÉSUMÈ -

In this study of teleost fin folds and scales, two important general points have emerged.

1. The investigation of early fin fold morphogenesis provides a clear example of a situation where apparently simple changes in tissue architecture involve co-operative interactions between a variety of distinctly different cellular mechanisms.

2. There is a close spatio-temporal correlation between cytoskeletal organisation, extracellular matrix deployment and cell shaping during development. This correlation may be a fundamental aspect of tissue organisation and may therefore be maintained in adult structures.

(a) Co-operative interactions between cellular mechanisms during fin fold morphogenesis

Ultrastructural and experimental analyses of fin fold morphogenesis indicate that conversion of a flat sheet of embryonic epidermis to a simple folded epithelium involves a complex series of changes in cell morphology. Changes in cell contact relation-

ships, shaping and proliferation or migration all play an important role in fin fold morphogenesis.

Similar mechanisms are involved in the morphogenesis of a wide variety of tissues and organs, for example, differential adhesion has been show to occur during limb bud development (Heintzelman, Phillips & Davis, 1978) and cell proliferation is important in the development of brain convolutions (Richman et al, 1975), whilst cell shape modulation has been shown in a variety of developmental systems (for review see Clarke & Spudich, 1977). It is interesting to note that many of the investigators involved in such analyses have indicated that a single force-generating mechanism predominates during morphogenesis of many of the tissues in question. Is there any reason why cells should not exploit all available mechanisms to achieve spatial precision during tissue shaping?

The spatio-temporal mechanics of any morphogenetic system which exploits more than one mechanism will be extremely complex. However, the involvement of several mechanisms may be crucial to ensure consistent success in forming complex body structures within the changing microenvironment of the developing embryo. For example, if only one mechanism is exploited during fin fold generation, then minor alterations in this mechanism may lead to major abnormalities in the adult fin. However, if the embryo adopts a "belt and braces - like" strategy in exploiting several mechanisms to achieve fin fold morphogenesis, then minor perturbations of any one mechanism may be compensated for by a continued utilisation of any of the other mechanisms which are involved.

(b) **The spatial correlation between the cytoskeleton and extracellular matrix in Brachydanio**

The experimental study of fin fold morphogenesis indicates that there is a fundamental correlation between cytoskeletal organisation and extracellular matrix deployment. However, several features of the tissue used in this study (for example thickness of the fin fold ectoderm and the lability of actin fibres) meant that the cytoskeleton could not be visualised at either the fine structural or light microscopic level. Fortunately, another tissue from Brachydanio provides one of the few opportunities to visualise the correlation between cytoskeletal organisation and extracellular matrix orientation in situ. The study of fibroblasts and osteoblasts on the scale of the adult teleost shows a close spatial correlation between the alignments of extracellular matrix components and cytoskeletal orientation in the fibroblasts. Furthermore, a previously unreported set of microtubules with an alignment which is correlated with extracellular topography was found in the osteoblasts. It is likely that such spatial correlations between intra- and extra-cellular components may be widespread and of fundamental importance in the control of cell shaping and function, not only during development, but also in apparently stable adult structures.

2. MORPHOGENESIS OF FIN FOLDS AND SCALES - PROSPECTS FOR FUTURE PROGRESS -

It might be particularly rewarding to assess the effect of specific substrates on morphogenesis of isolated cell monolayers taken from putative and mature fin fold epidermis cultured in vitro. For example, aligned collagen matrices could be used in attempts to assess whether the deposition of parallel arrays of extracellular fibres (in particular, actinotrichia) may be involved in subsequent epidermal cell flattening and fin fold expansion. Furthermore, the impact of fibronectin-rich and fibronectin-free culture media and substrates could be compared to monitor how this important cell adhesion molecule can influence apical ectodermal ridge development and its subsequent conversion to a fin fold.

The correlation between cytoskeletal orientations and the patterns of extracellular matrix deposition in cyprinid scales provides a number of exciting possibilities for future research. For example, it would be particularly valuable to investigate how this correlation is established in developing scales. Such studies might indicate how decentralised control of microtubule bending is achieved and whether the orientation of extracellular matrix components does have a significant impact on cytoskeletal organisation (or vice-versa).

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Fig. 13

Stage 19. Differential interference contrast micrograph of a tail bud showing the ectoderm (arrow) which is composed of peridermis and underlying epidermis. x 500.


13

Fig. 14

Early stage 20. Cross section through a developing apical ectodermal ridge showing the arrangement of its wedge-shaped epidermal cells (between arrows) with respect to the overlying peridermis and underlying mesoderm. x 5000.

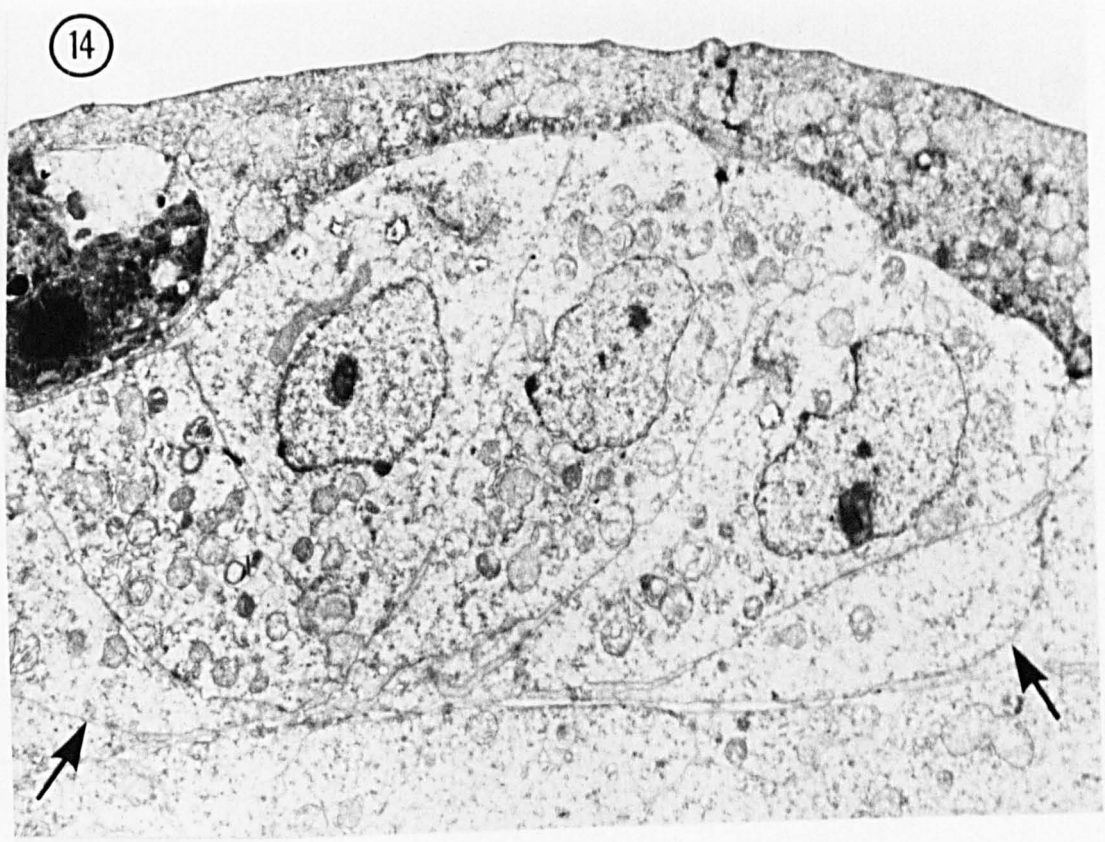
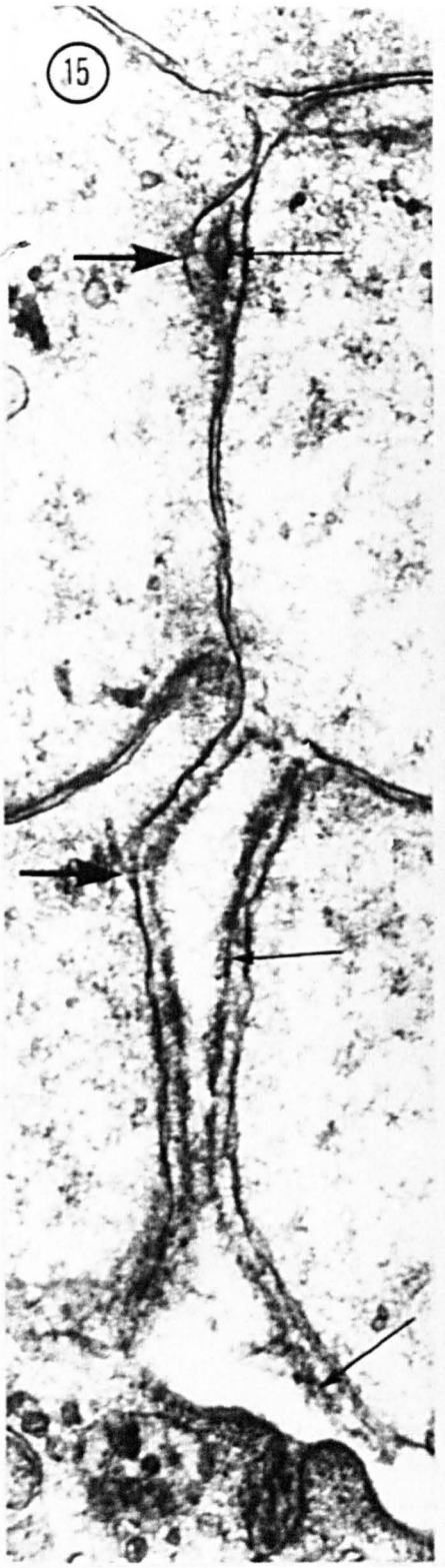

14

Fig. 15

Mid stage 20. Cross section through a portion of an ectodermal ridge showing discrete extracellular spaces (large arrows) between the basal surfaces of adjoining epidermal cells flanking the sides of the ridge. These spaces are lined by a basal lamina (small arrows). Osmium-ferricyanide fixations. x 36000.

Fig. 16

Mid stage 20. Cross section through a portion of a developing ectodermal ridge showing the contribution of a cell surface invagination to one of the extracellular spaces forming between the basal surfaces of neighbouring epidermal cells. Numerous vesicles (V) and electron dense cytoplasm are concentrated around the invaginated cell surface region. The basal lamina is connected to cell surface membranes by small dense bridges (large arrows) and granular extracellular material (small arrows) occurs between apposed basal laminae. x 81000.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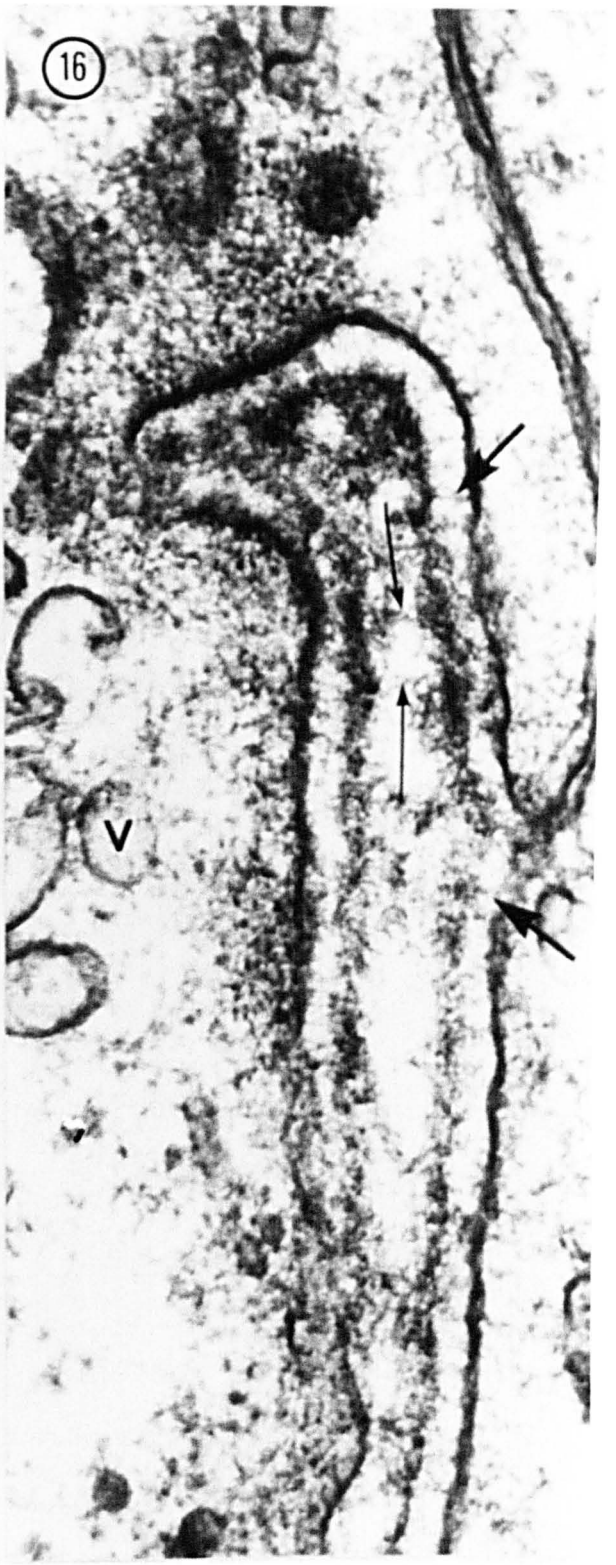

16
V

Fig. 17

Late stage 20. Cross section through an early fin fold. The subepidermal space (arrows) extends from the invagination in a cleft cell (C) to the base of the fin fold. Osmium-ferricyanide fixation. x 6600.

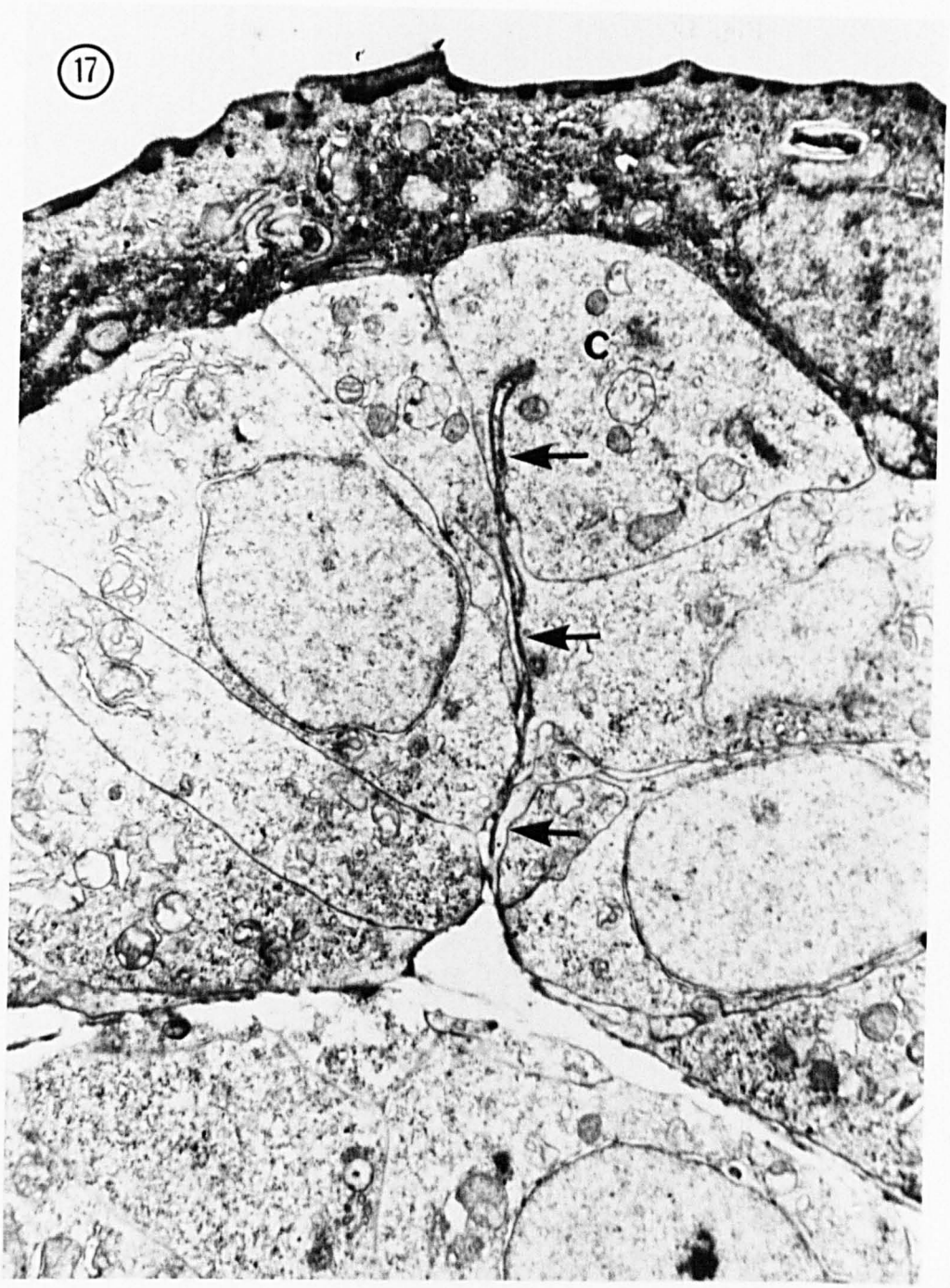

17
C

Fig. 18

Stage 21. Cross section of a cleft cell invagination showing cross-sectional profiles (small arrows) of portions of actinotrichia that are oriented parallel to the longitudinal axis of the fin fold. Actinotrichia run proximo-distally towards the base of the fold (large arrow). x 53000.

Fig. 19

Stage 21. Longitudinal section through a portion of an early fin fold cut in a plane at right angles to the plane of the fold and grazing through the top of the subepidermal space where it is capped by cleft cell invaginations (I). Adjacent cleft cells overlap and interdigitate (see region between arrows). In such regions two cleft cells contribute to the invagination. Osmium-ferri-cyanide fixation. x 31000.

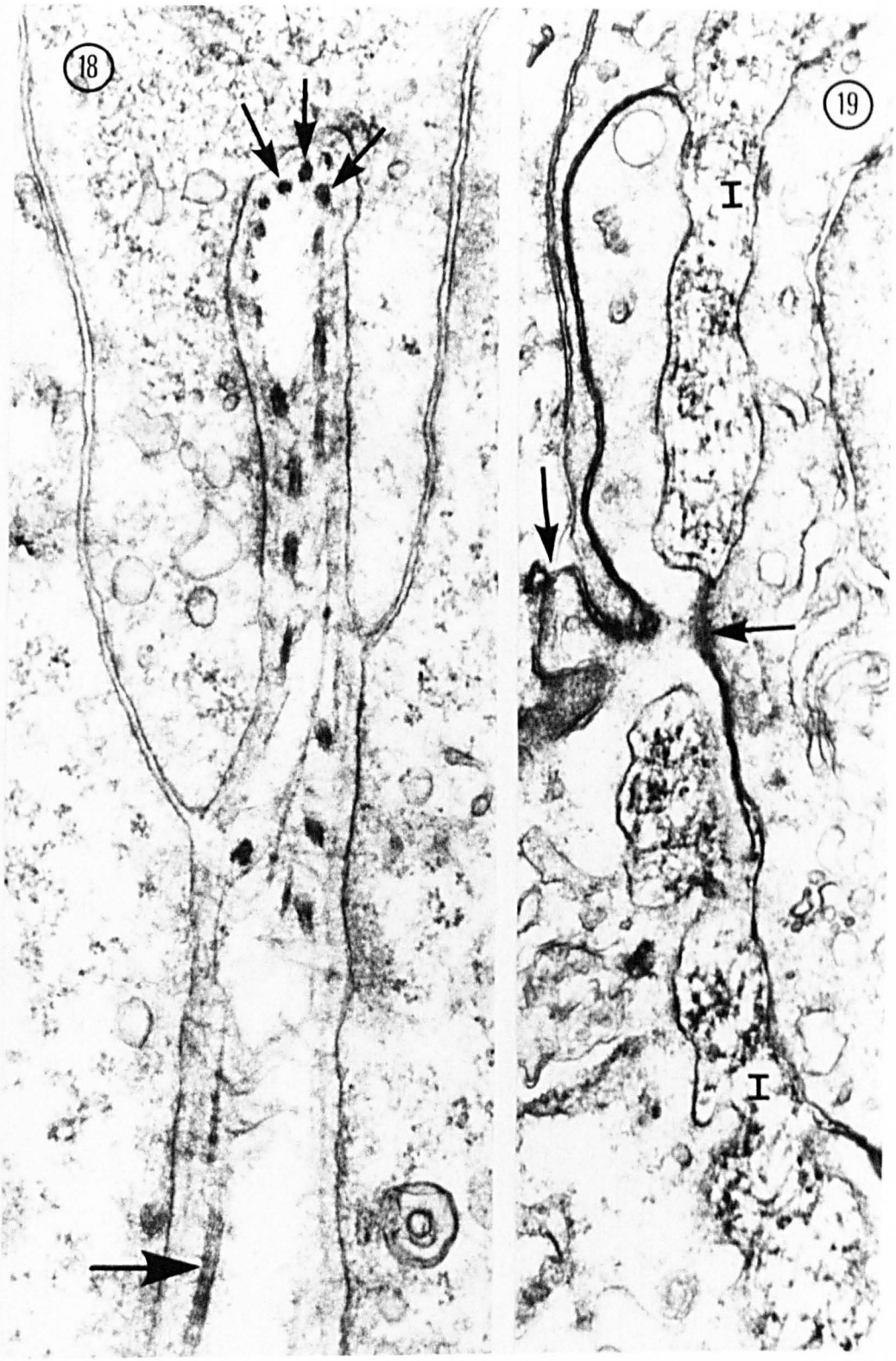

18
19
I
I

Fig. 20

Early Stage 21. Cross section through a portion of an early fin fold. Numerous extracellular cross fibres (small arrow) that are associated with dense granular matrix material (large arrow) span the subepidermal space. Osmium-ferricyanide fixation. x 19500.

Fig. 21

Mid stage 21. Longitudinal section through a portion of a fin fold cut at right angles to the plane of the fold showing two rows of proximo-distally oriented actinotrichia (A) on either side of the subepidermal space. A few cross fibres remain. Some of the ends of these fibres (large arrow) penetrate the basal lamina (small arrows) and contact the surface membranes of epidermal cells. Osmium-ferricyanide fixation. x 91000.

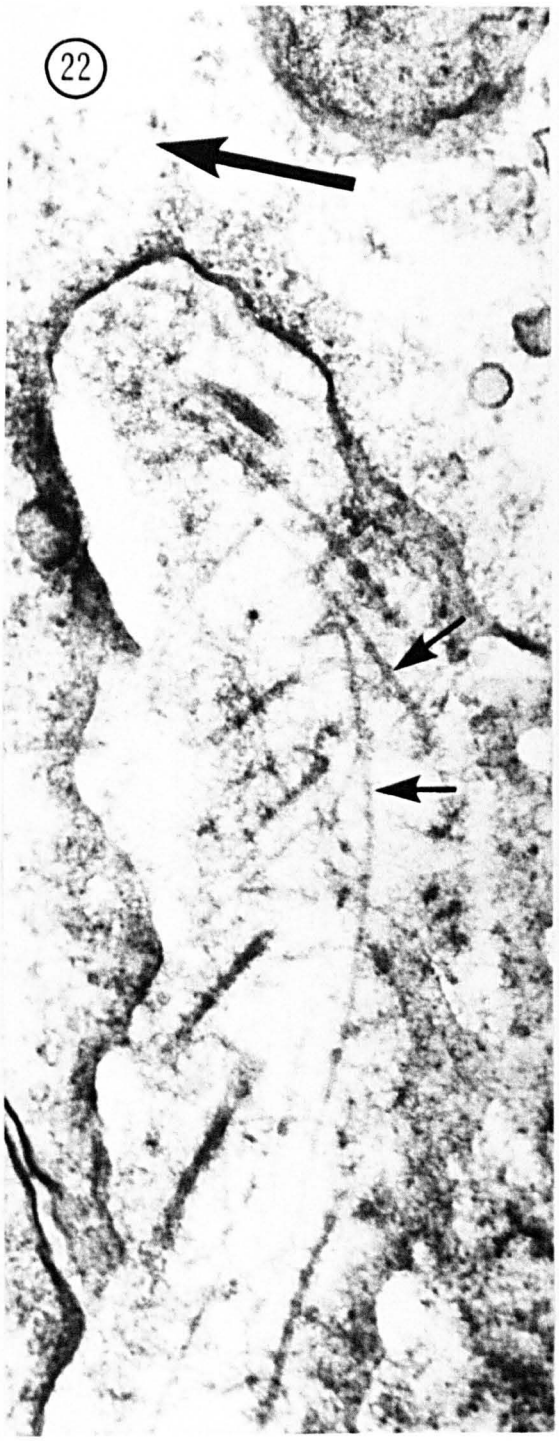

20
21
A

Fig. 22

Mid stage 21. Longitudinal section through a portion of a cleft cell invagination cut in a plane parallel to the plane of the fin fold showing posteriorly curving portions of actinotrichia (small arrows) which occur towards the apex of the subepidermal space (large arrow points posteriorly). x 68000.

Fig. 23

Late stage 21. Section grazing through the basal surface of an epidermal cell flanking the sides of a fin fold. Bundles of intermediate filaments (arrows) approach cell surface regions that are positioned close to actinotrichia (A) in the subepidermal space. x 68000.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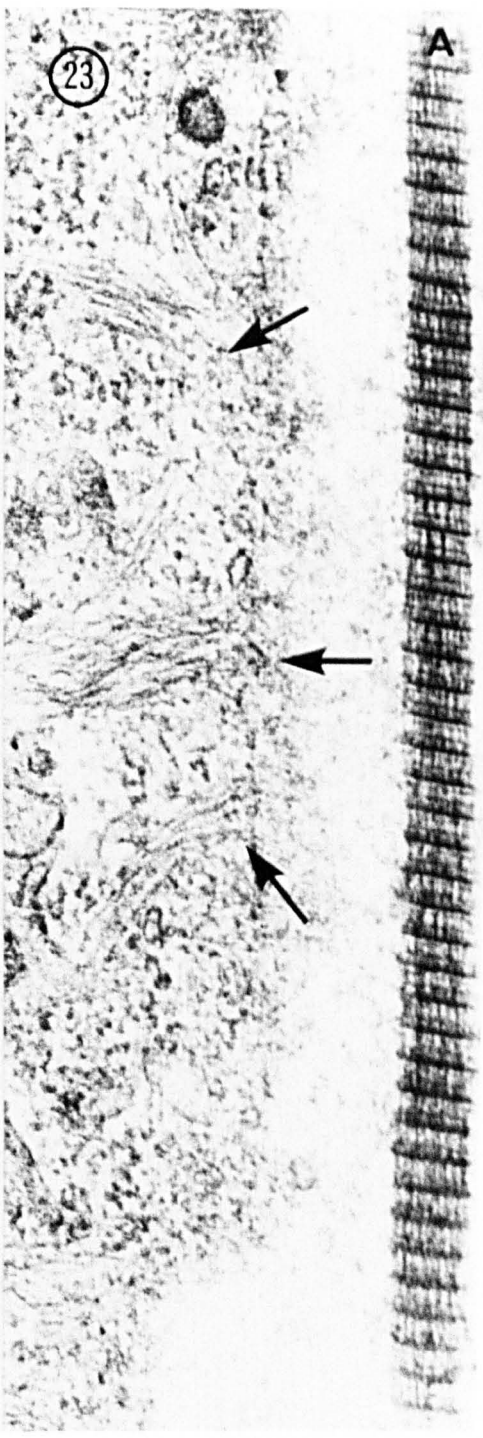

23
A

Fig. 24

Stage 23-24. Differential interference contrast micrograph showing part of the caudal fin fold of Brachydanio (large arrows). The flattened lamella-like fin fold contains large numbers of migratory mesodermal cells (small arrows). x 500.

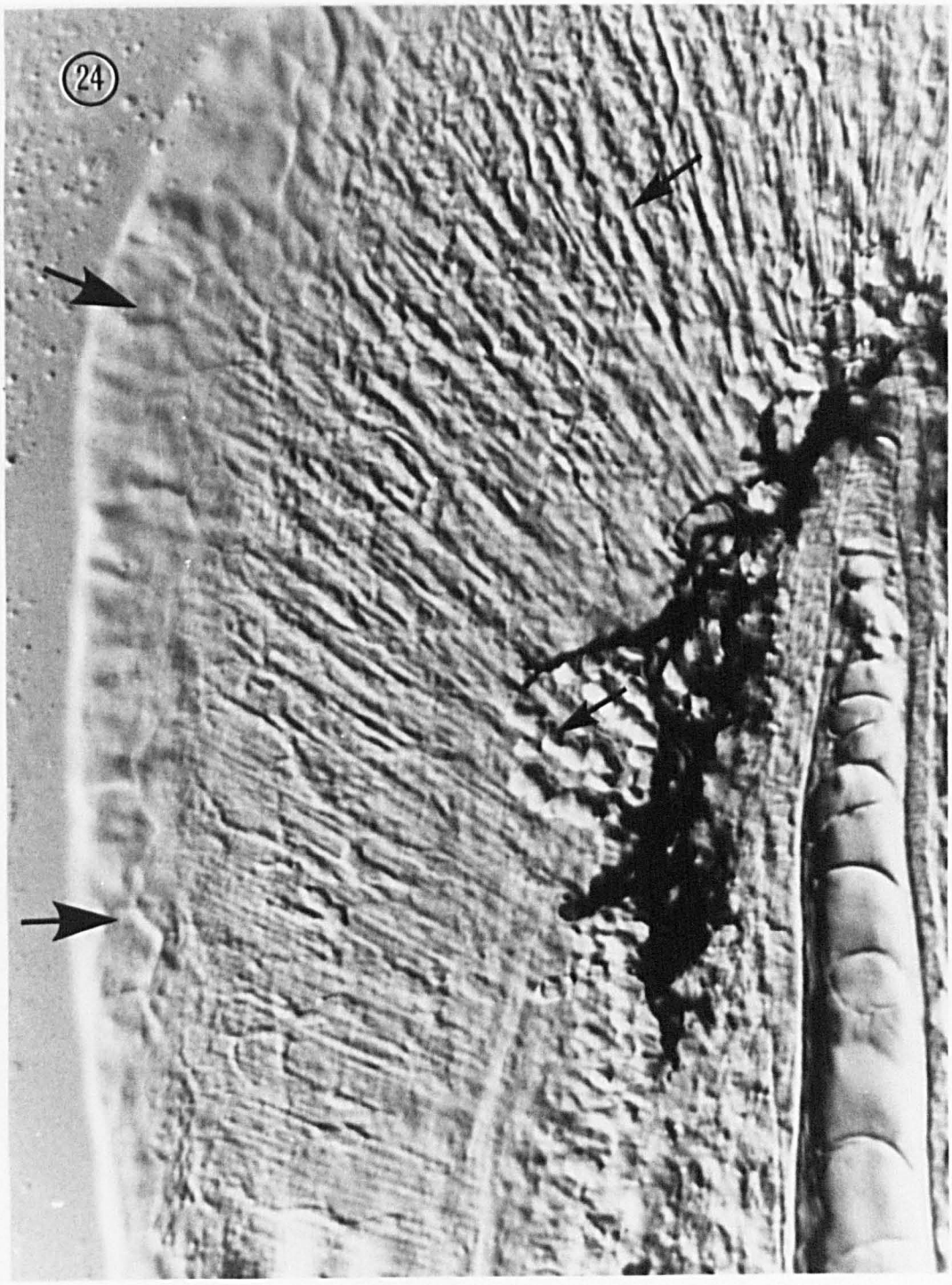
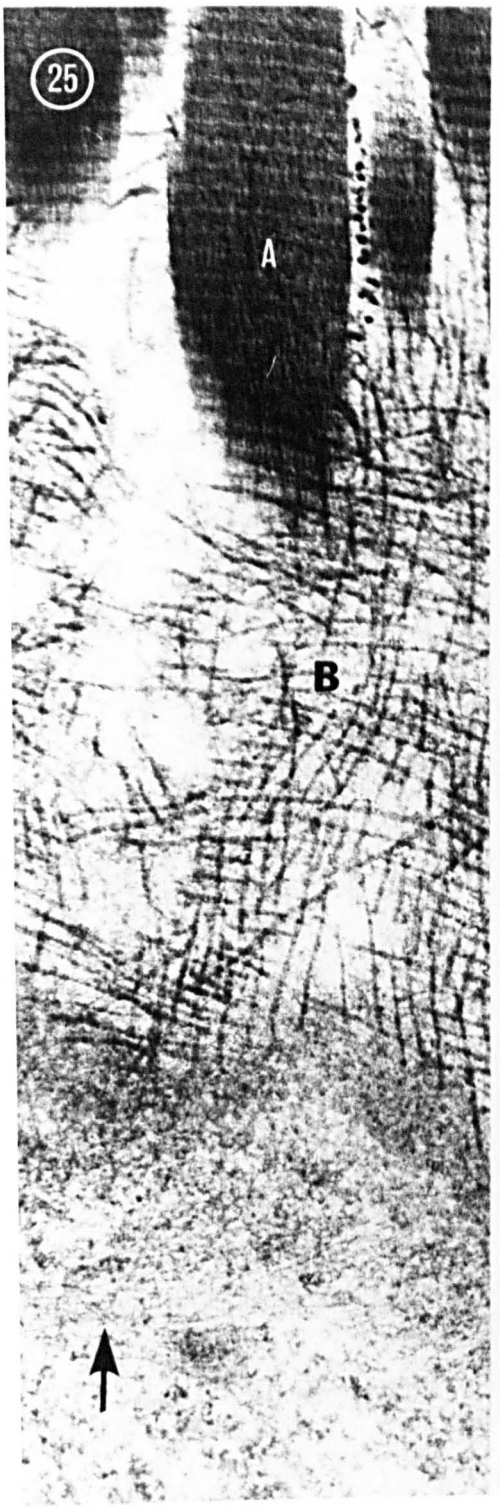

24

Fig. 25

Stage 24. Section grazing through the basal surface of a fin fold epidermal cell. A basement lamella (B) includes an array of orthogonally arranged collagen fibres. This array is interposed between actinotrichia (A) and the basal lamina. A layer of intermediate filaments (arrow) is juxtaposed against the cytoplasmic side of the cell surface. x 46000.

Fig. 26

Stage 24. Section grazing through a fin fold epidermal cell. Extensive layers of intermediate filaments (arrows) ramify throughout the cytoplasm and are associated with densely staining bodies (X) close to the cell surface. x 55000.


25
A
B

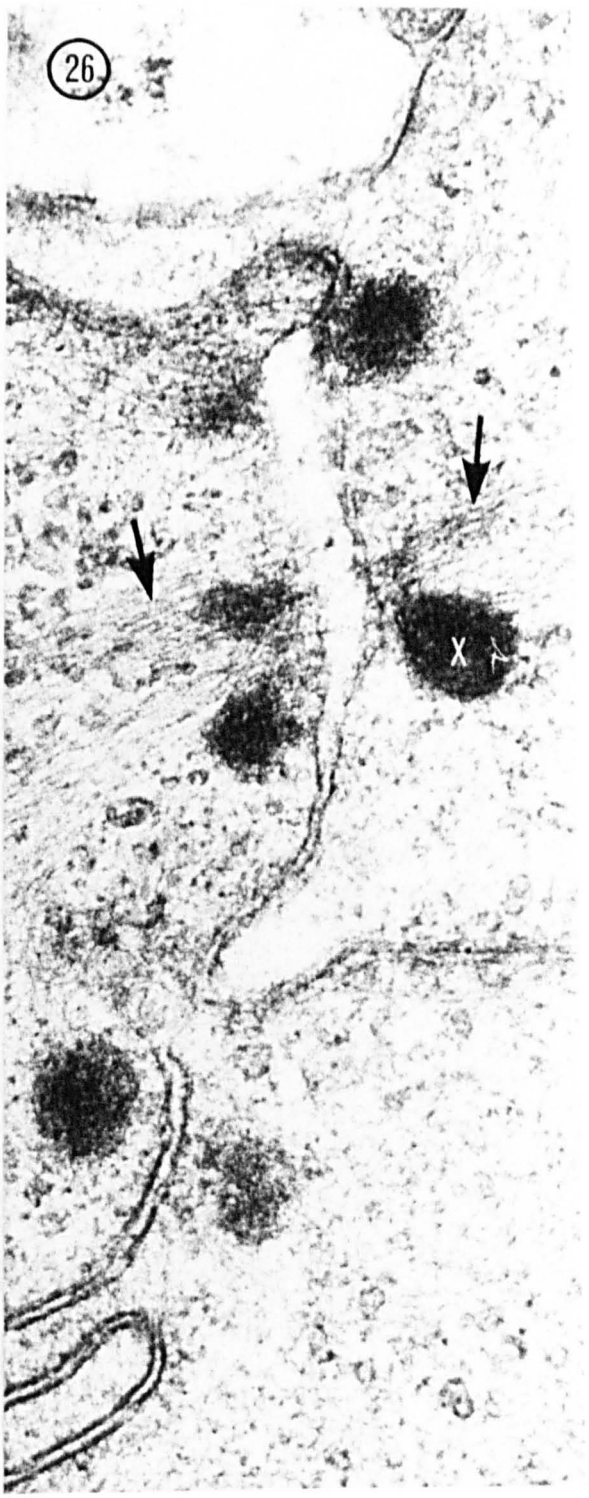

26
X

Fig. 27

Stage 24. Cross section through a central portion of a fin fold. The subepidermal space is bounded by a flattened layer of epidermal cells (E) overlain by peridermis (P). Mesodermal cells (M) span the subepidermal space and migrate in close proximity to actinotrichia (arrows). x 11500.

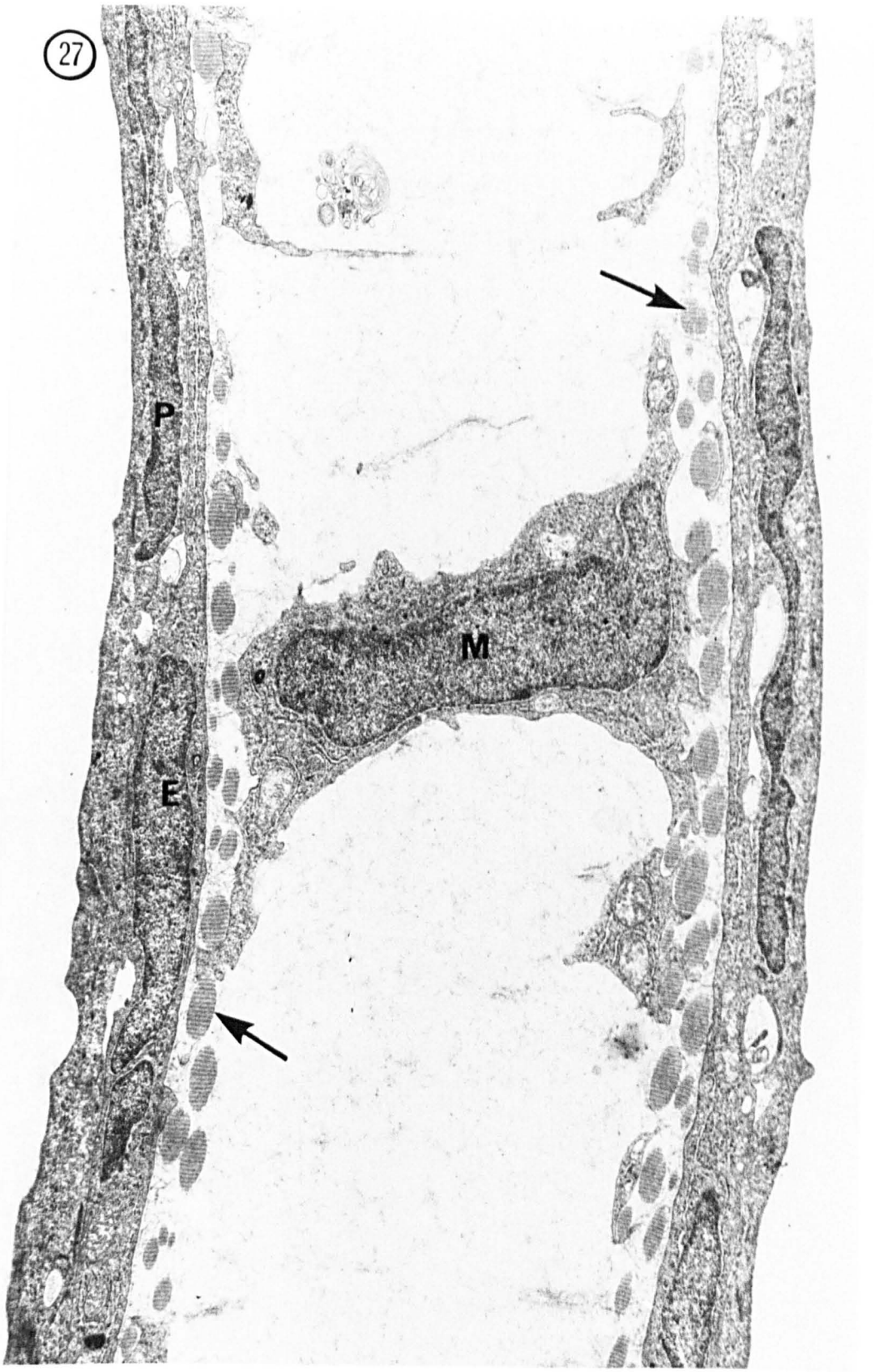

27
P
M
E

Fig. 28

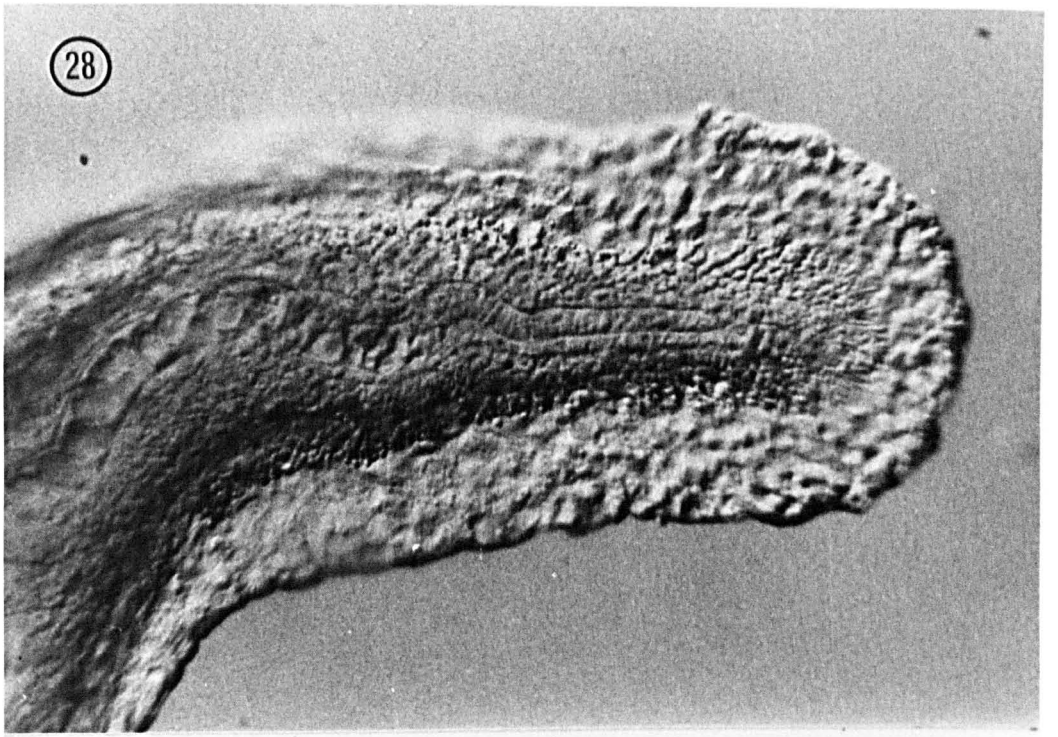
Differential interference contrast micrograph of a tail bud which has been cultured *in vitro* for 24 hours from stage 19. The fin fold shows few developmental abnormalities (compare with Fig. 24). x 260.

Fig. 29

Cross section through a central portion of the fin fold illustrated in Fig. 28 showing parallel arrays of actinotrichia (arrows) similar to those observed in the subepidermal spaces of fin folds which developed *in vivo*. x 45000.

Fig. 30

Cross section through an apical portion of a fin fold similar to that illustrated in Fig. 28 showing cross sectional profiles of portions of actinotrichia (arrow) which are located close to the cleft cell invagination. x 38000.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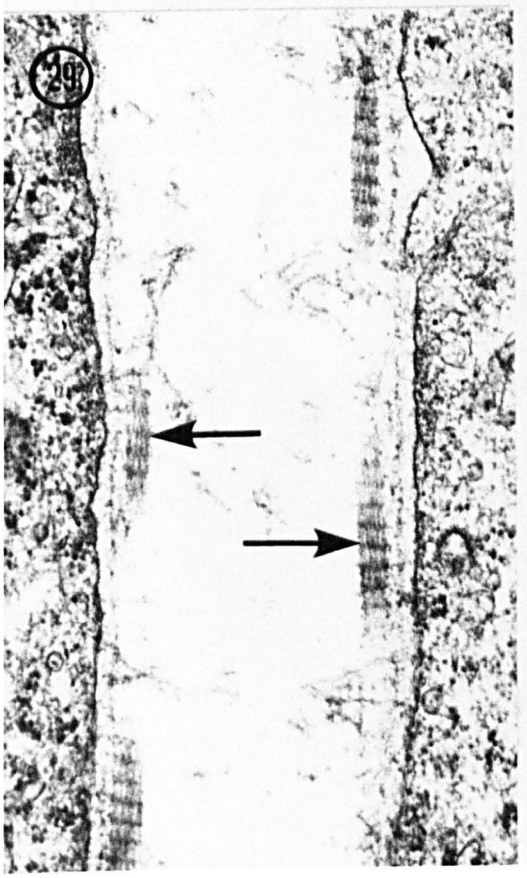
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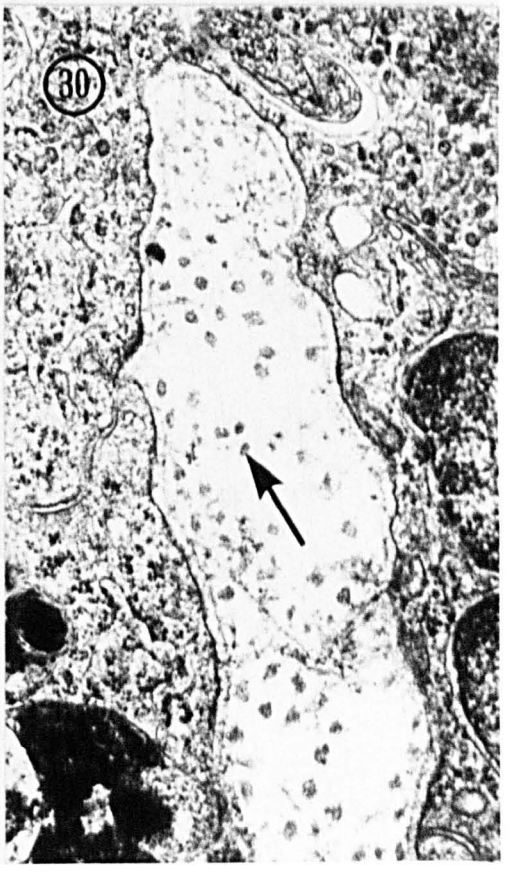
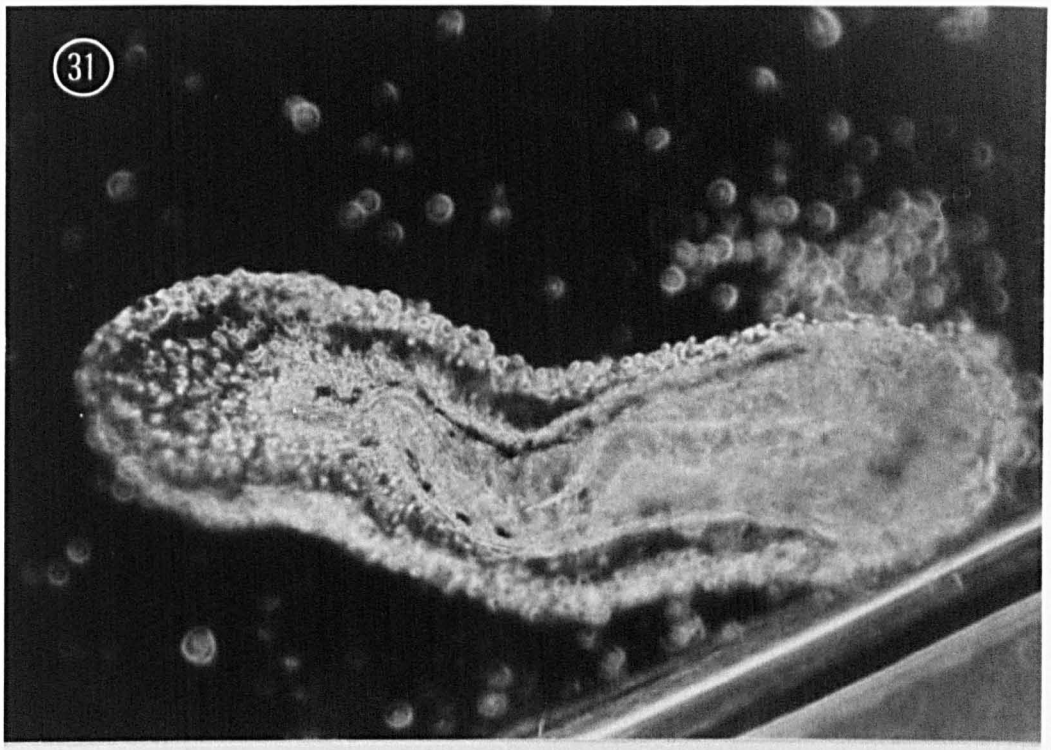

30

Fig. 31

Dark field micrograph of a tail bud which has been cultured for 96 hours *in vitro* from stage 19. The tail bud and fin fold are contorted and mishapen, unlike those which have developed for a period of less that 72 hours *in vitro* (compare with Fig. 28). x 140.

Fig. 32

Cross section through a portion of the fin fold from the tail bud illustrated in Fig. 31 showing how actinotrichia (arrows) lose their alignment after a period of more than 72 hours *in vitro*. x 68000.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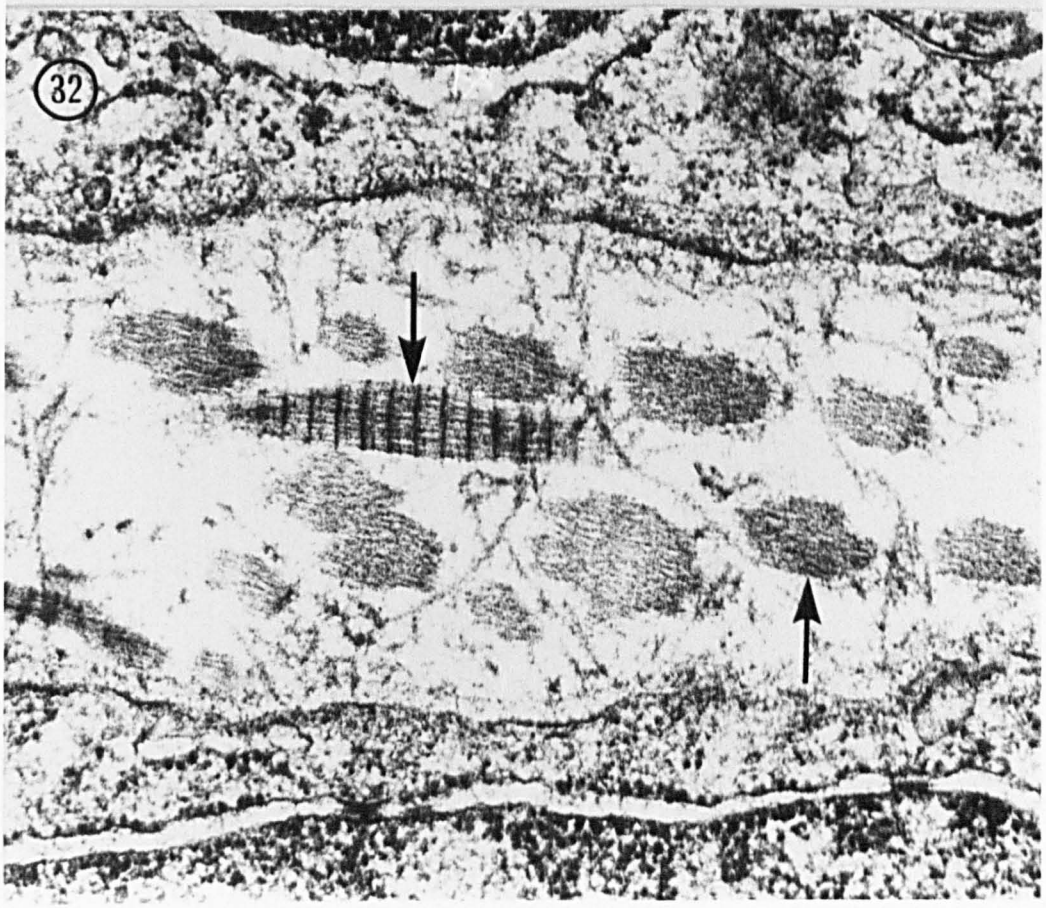

32

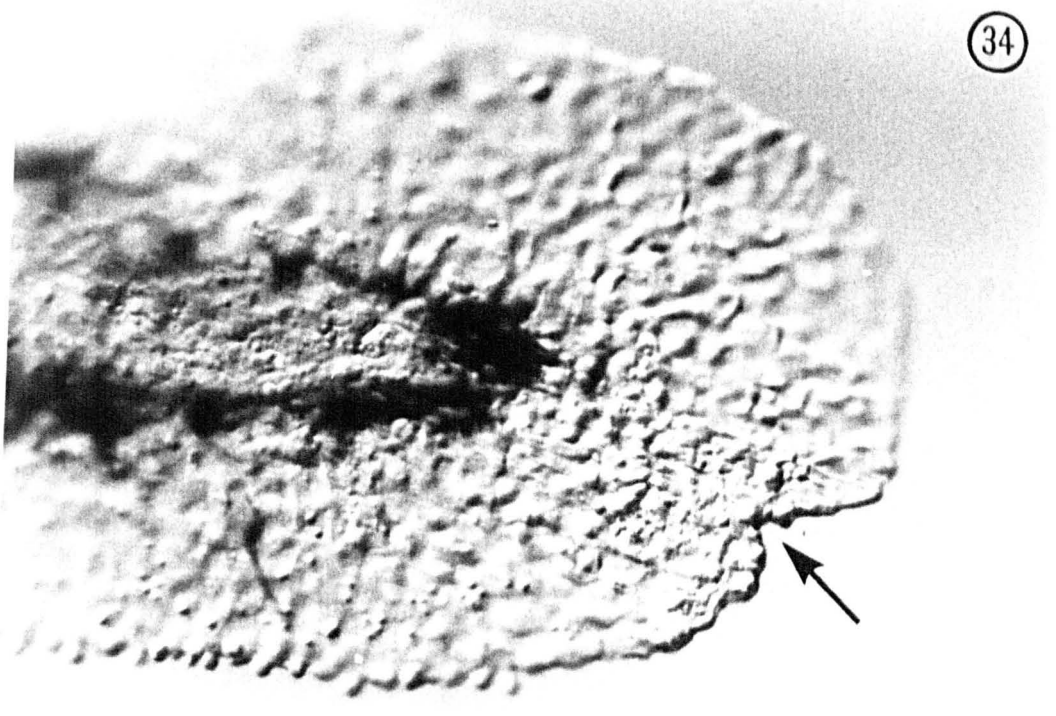
Fig. 33

Differential interference contrast micrograph of an isolated tail bud. An apical portion of the early fin fold was removed (arrow) by microsurgery 10 mins before the tail bud was photographed. x 350.

Fig. 34

The same tail bud as in Fig. 33 after 48 hours in culture. The amputated region (arrow) has not been replaced and is still located at the distal margin of the fold. x 350.

33



34

Fig. 35

Cross section through the fin fold of a tail bud similar to that illustrated in Fig. 34. The subepidermal space (S) is swollen and "balloon-like". Actinotrichia (arrows) are disoriented and mesodermal cells (M) have not migrated into apical portions of the fold. x 7000.

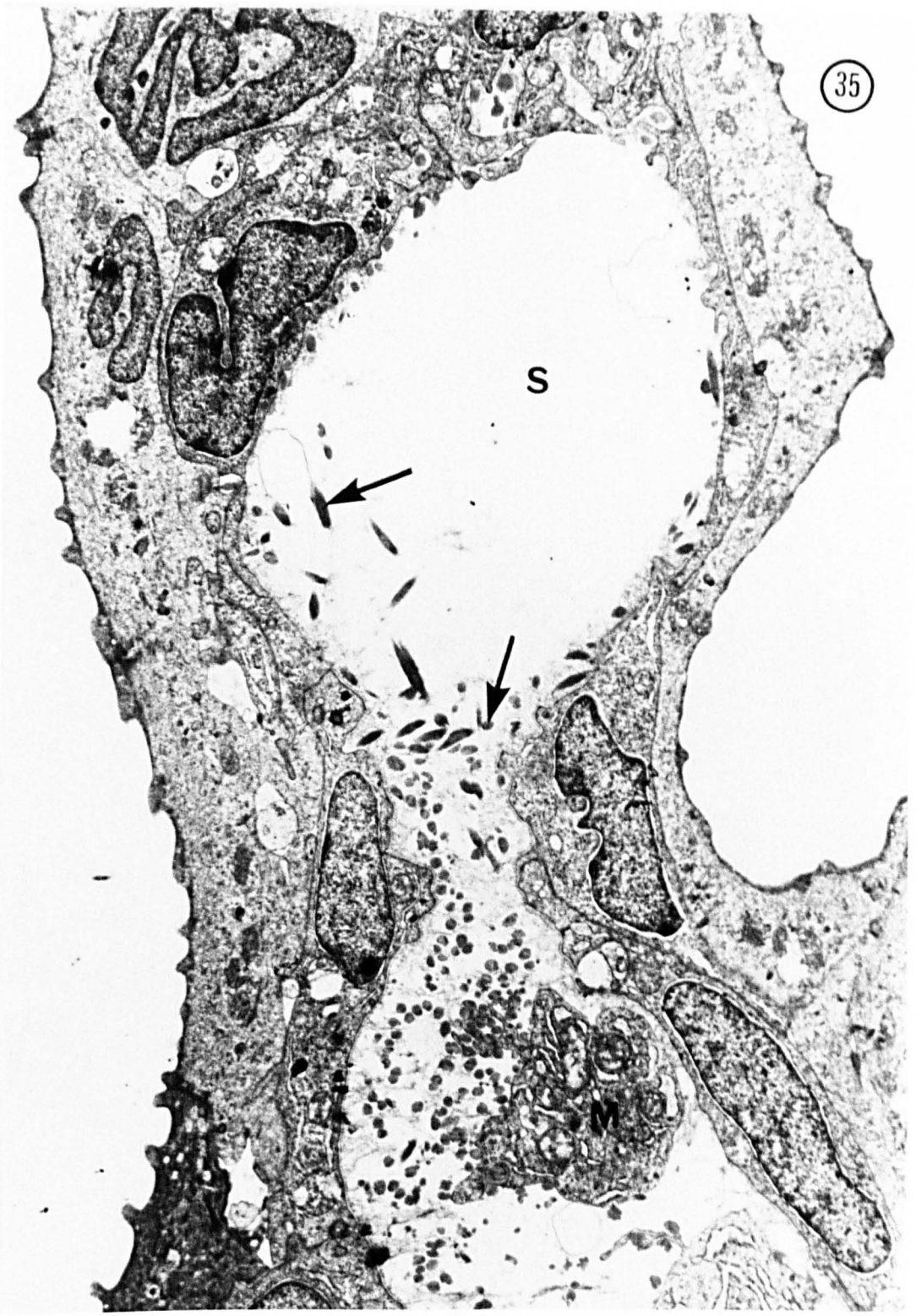
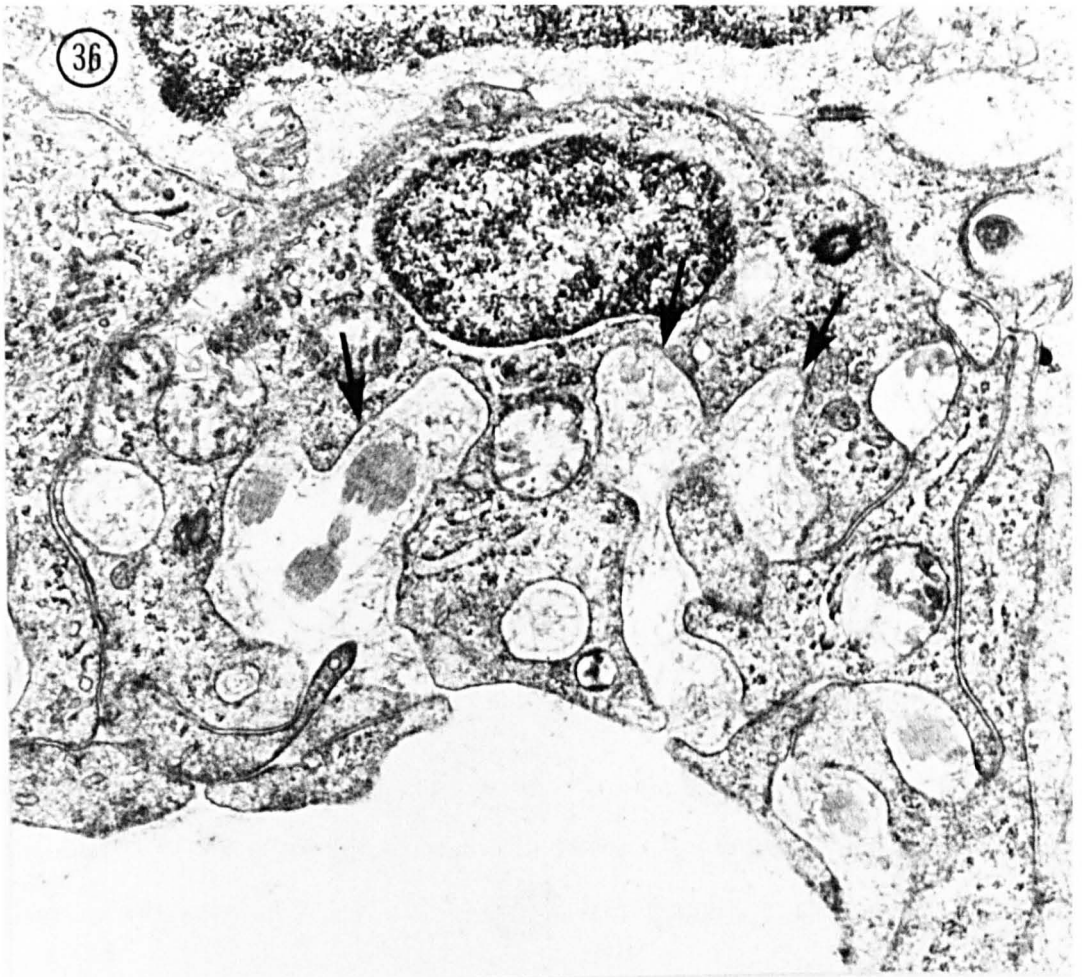

35
S
M

Fig. 36

Cross section through an epidermal cell near to the apex of a fin fold similar to that illustrated in Fig. 35 showing numerous invaginations (arrows) in the basal surface of the cell. x 22000.

Fig. 37

Cross section through the ectoderm of a tail bud at the site of microsurgery 48 hours after the complete fin fold had been removed. There are no recognisable fin fold-like structures and a flattened layer of epidermis (E) is overlain by peridermis (P). x 8700.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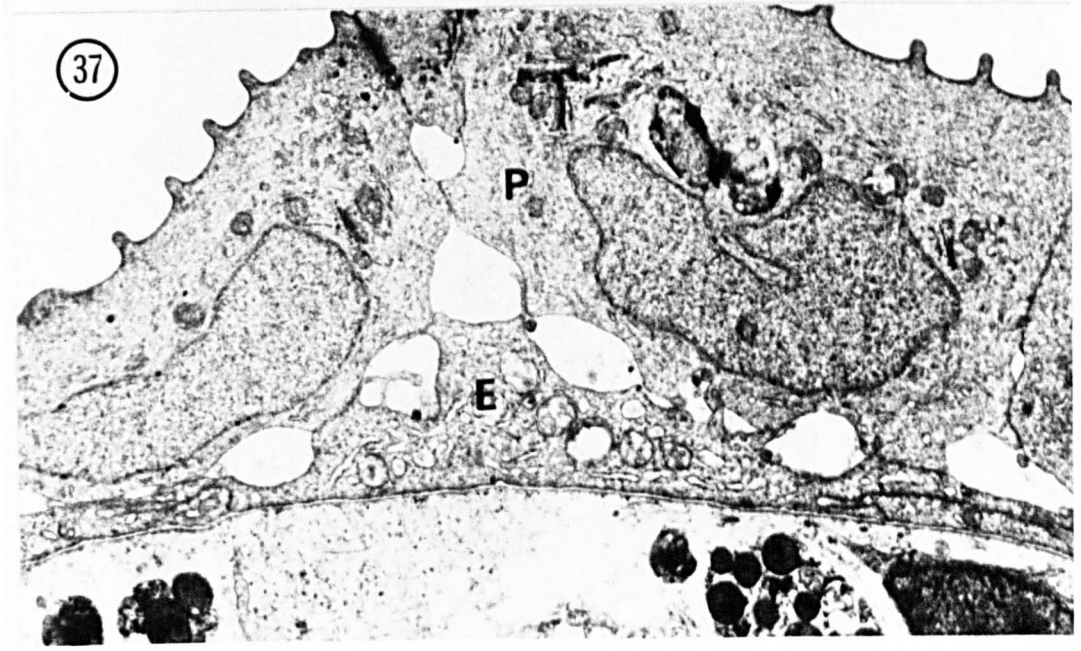
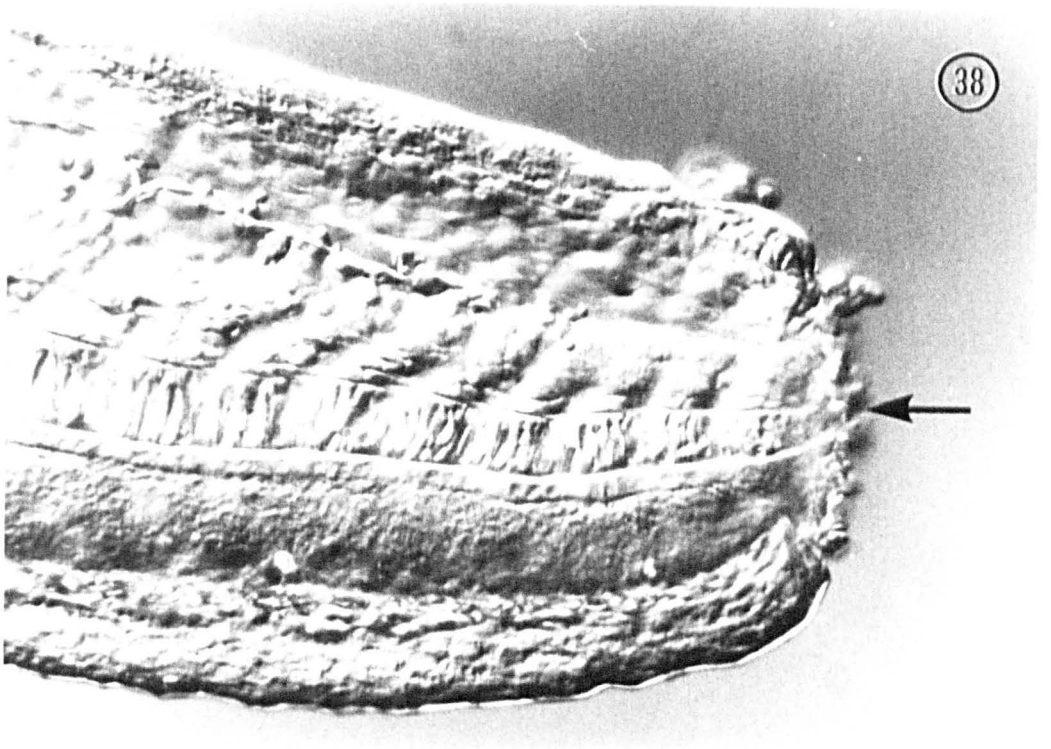

37
P
E

Fig. 38

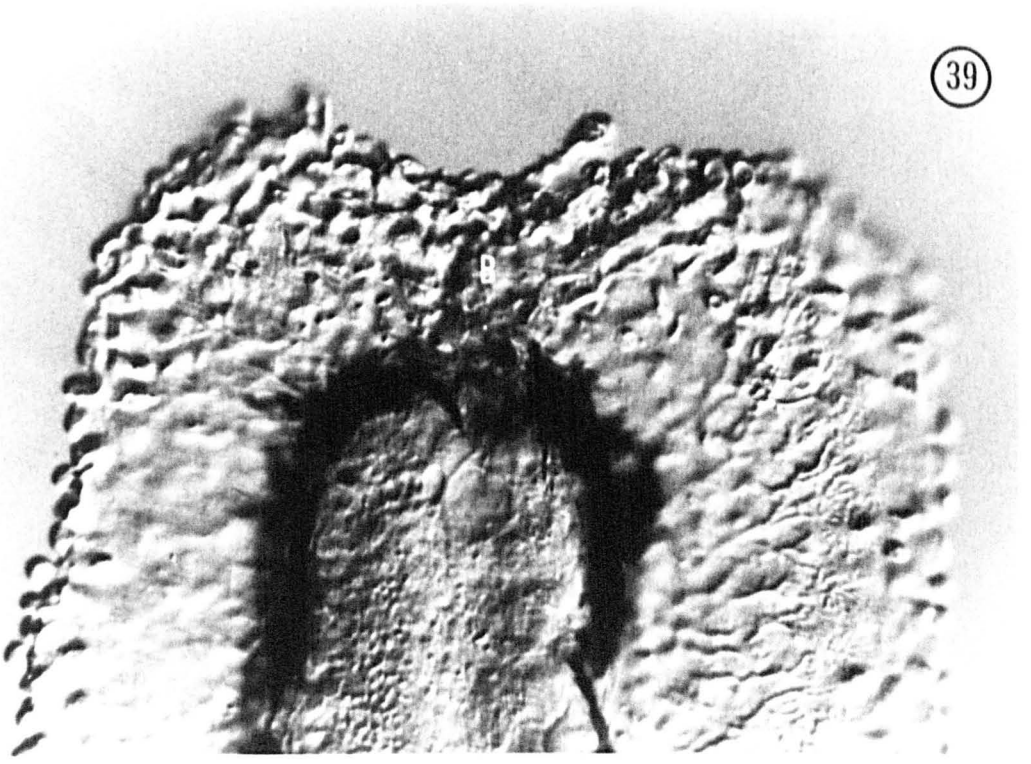
Differential interference contrast micrograph of an isolated tail bud immediately after a portion of a fin fold and underlying mesoderm had been removed by microsurgery (amputation face is arrowed). x 400.

Fig. 39

The same tail bud as that illustrated in Fig. 38 after 24 hours in culture. A wound blastema (B) has formed at the site of amputation. x 300.



38



39

Fig. 40

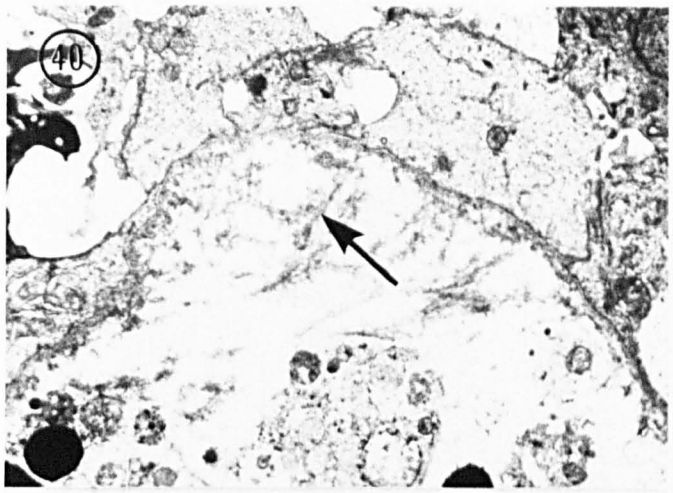
Cross section through a portion of the blastema of a tail bud similar to that described in Fig. 39. After 20 hours in culture a small extracellular space which contains fibrillogranular matrix (arrow) is located at the base of the blastema. x 6900.

Fig. 41

Cross section through a portion of the blastema of a tail bud similar to that described in Fig. 39. After 24 hours in culture the extracellular space (S) has enlarged distally and resembles the subepidermal space of a normal fin fold. x 7700.

Fig. 42

Cross section through a portion of a blastema similar to those described in Figs. 40 and 41. After 60 hours in culture the extracellular space is contorted and contains numerous disoriented actinotrichia (arrows). x 28000.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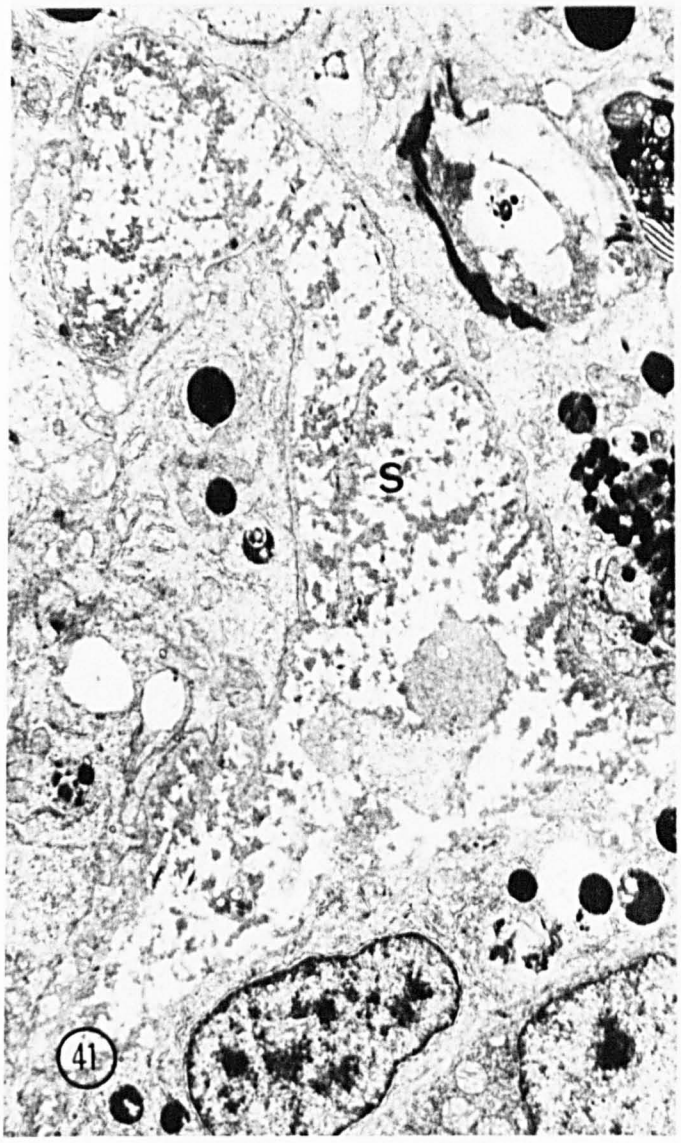

S
41


42

Fig. 43

Cross section through a portion of putative fin fold epidermis from a tail bud which had been exposed to cytochalasin B for 3 hours during the stage of apical ectodermal ridge construction. A disorganised cluster of putative fin fold epidermal cells (between arrows) is associated with the tail bud whilst the remaining epidermis (E) and overlying peridermis have sloughed away. x 4700.

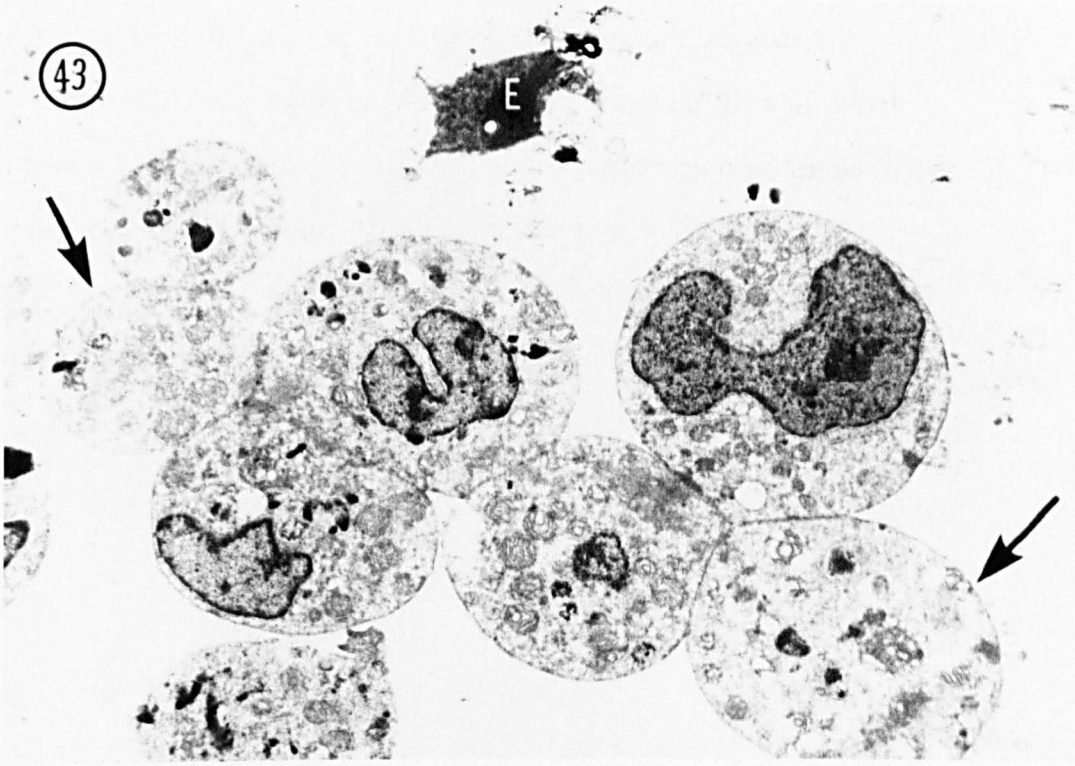

43
E

Fig. 44

Cross section through a portion of an epidermal cell from a fin fold-like structure which has developed on a tail bud which was exposed to cytochalasin B as described for Fig. 43 and then left to recover for 24 hours in cytochalasin-free culture medium. A cleft-like invagination (C) has formed along the basal surface of the cell. x 19000.

Fig. 45

Cross section through a fin fold-like structure similar to that described in Fig. 44 showing the subepidermal space (arrows) and underlying mesoderm (M). x 5500.

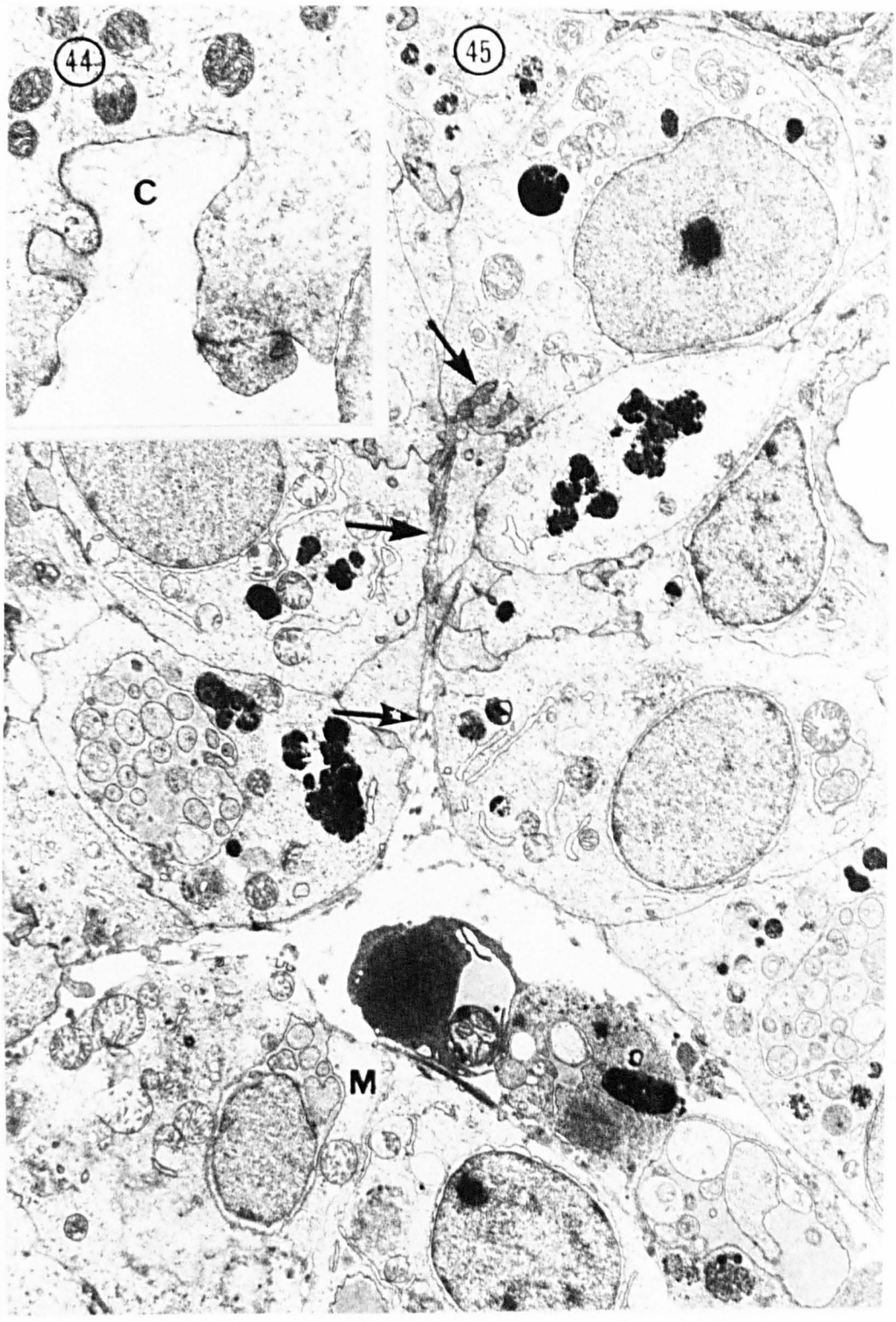
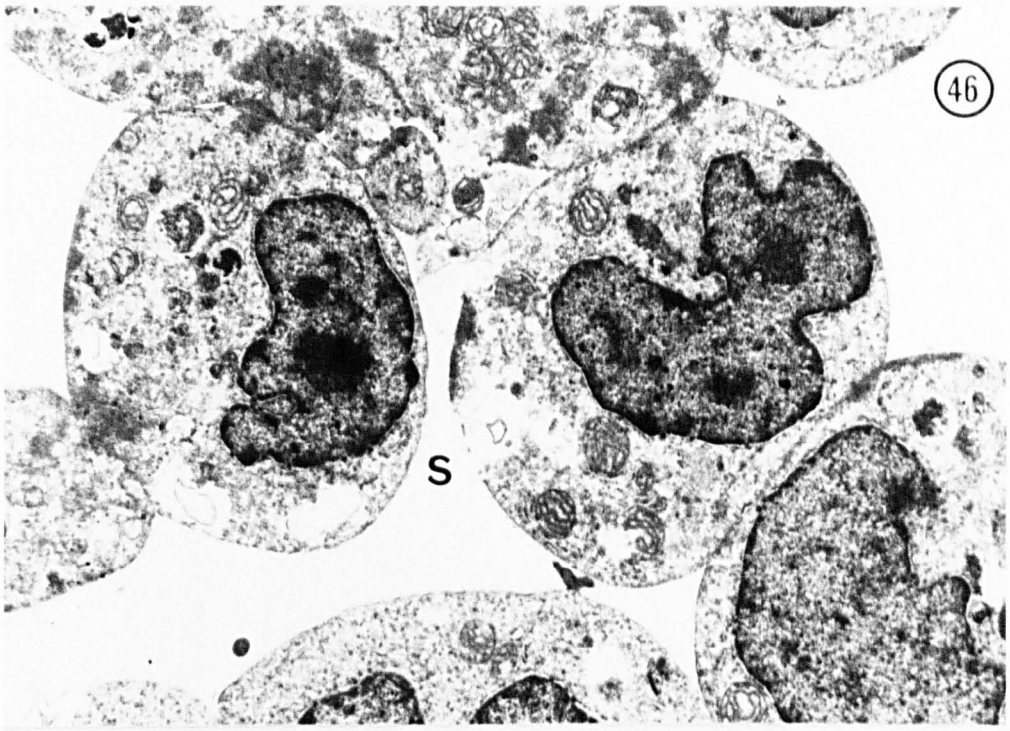

44
C
45
M

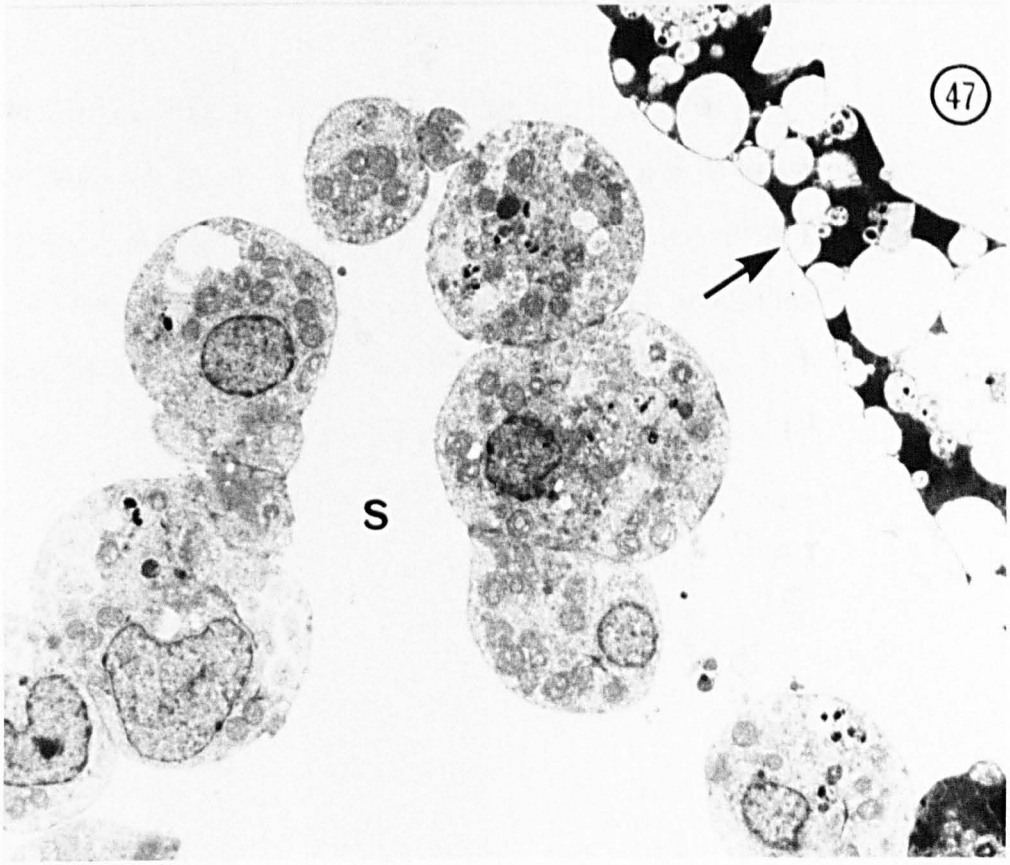
Fig. 46

Cross section through a portion of fin fold epidermis from a tail bud which had been exposed to cytochalasin B for a period of 6 hours during the stage of apical ectodermal ridge construction and its subsequent conversion to an early fin fold. An extracullular space (S) is located in the centre of a cluster of irregularly shaped epidermal cells. x 8000.

Fig. 47

Cross section through a portion of putative fin fold epidermis from a tail bud which had been exposed to cytochalasin B for 1 hour longer than that illustrated in Fig. 46. Irregularly shaped epidermal cells flank a large central extracellular space (S). The remainder of the tail bud epidermis and overlying peridermis (arrow) have sloughed away. x 4300.


46
S


47
S

Figs. 48 and 49

Cross sections through epidermal cells similar to those described in Fig. 46. Large numbers of microtubules (large arrows) are located close to the cell surface membrane (small arrows) x 88000 (Fig. 48).
x 120000 (Fig. 49).

Fig. 50

Cross section through an epidermal cell similar to one of those illustrated in Fig. 46 showing microtubular profiles surrounded by a cell membrane. This structure resembles a primary cilium. x164000.

Fig. 51

Cross section through an epidermal cell similar to one of those illustrated in Fig. 47 showing a centrosome and an associated cross sectional profile of a cytoplasmic microtubule (arrow). x 107000.

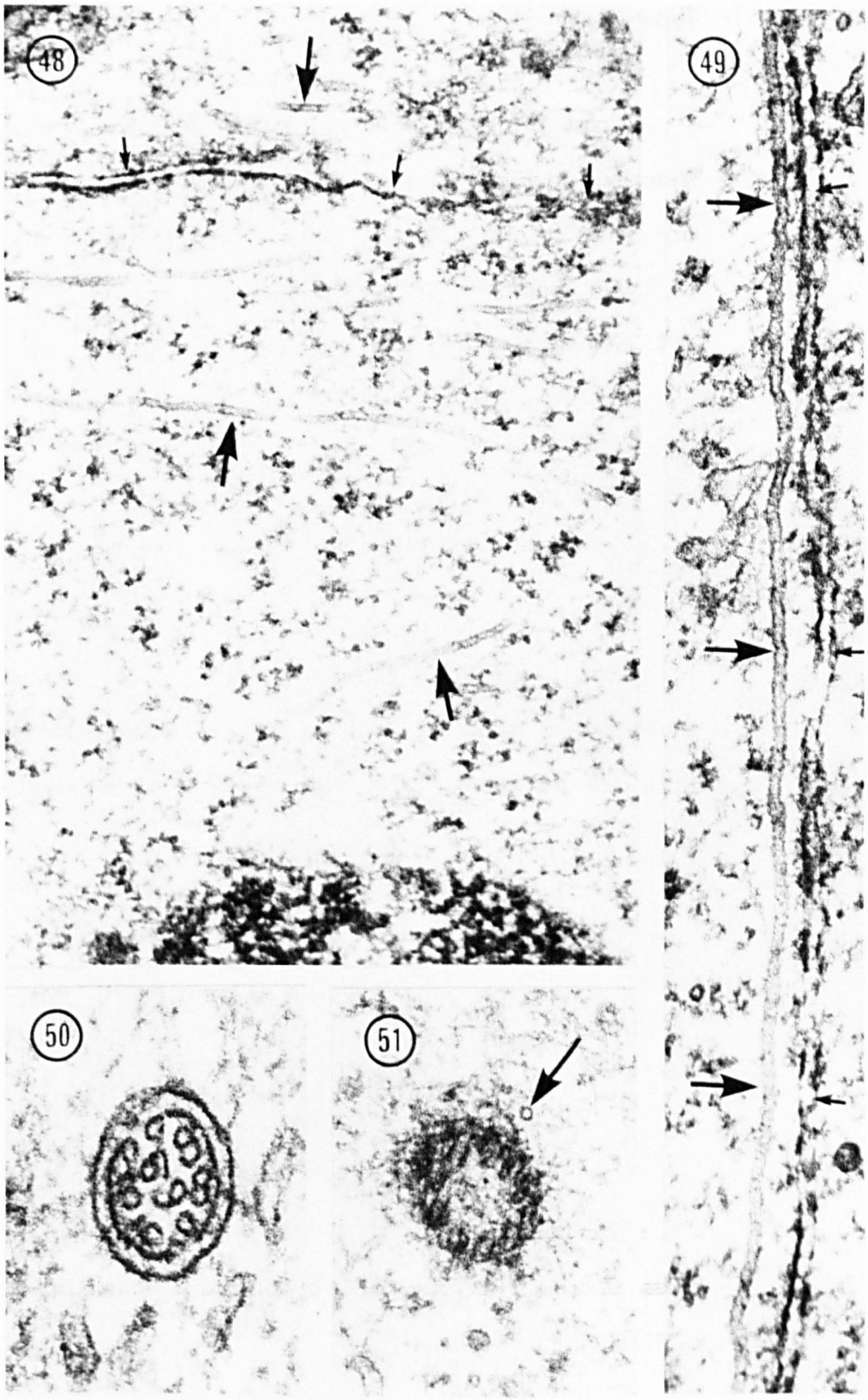

48
49
50
51

Fig. 52

Cross section through a fin fold from a tail bud which had been exposed to cytochalasin for 3 hours during stage 21 and then left to recover for 18 hours. The subepidermal space (S) is swollen and numerous extracellular spaces (arrows) have developed between adjacent epidermal and peridermal cells. x 6800.

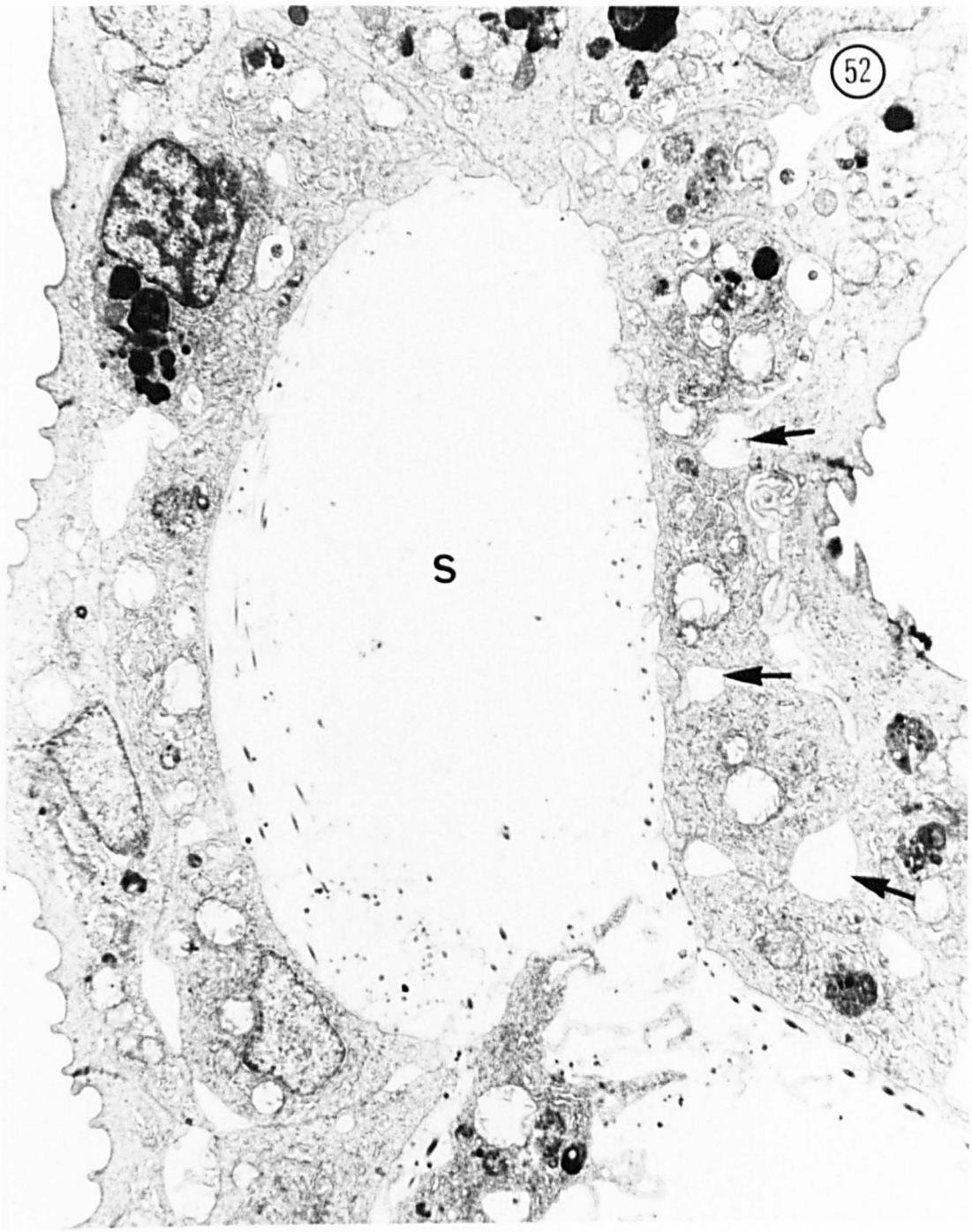
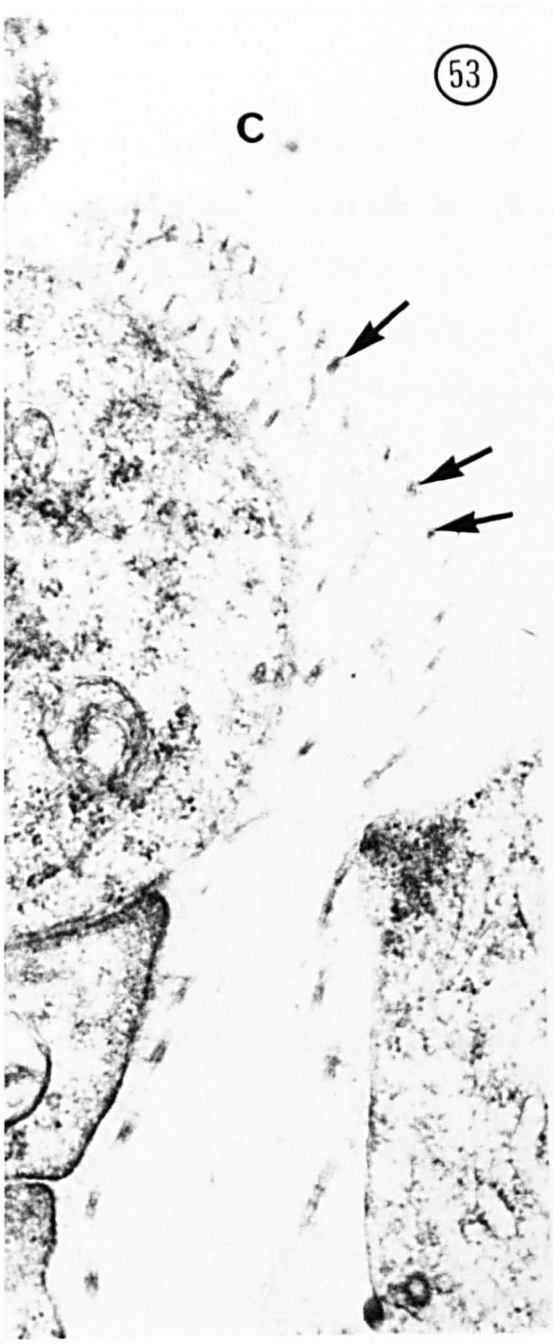

52
S

Fig. 53

Cross section through an apical portion of a fin fold similar to that illustrated in Fig. 52 showing cross sectional profiles of longitudinally oriented actinotrichia (arrows) which retain their anterior-posterior orientation although the cleft cell has disintegrated. (C indicates region of cleft cell before disintegration). x 42000.

Fig. 54

Cross section through a proximal portion of a fin fold similar to that illustrated in Fig. 52 showing disoriented arrays of actinotrichia (arrowed) within the proximal subepidermal space. x 39000.


53
C

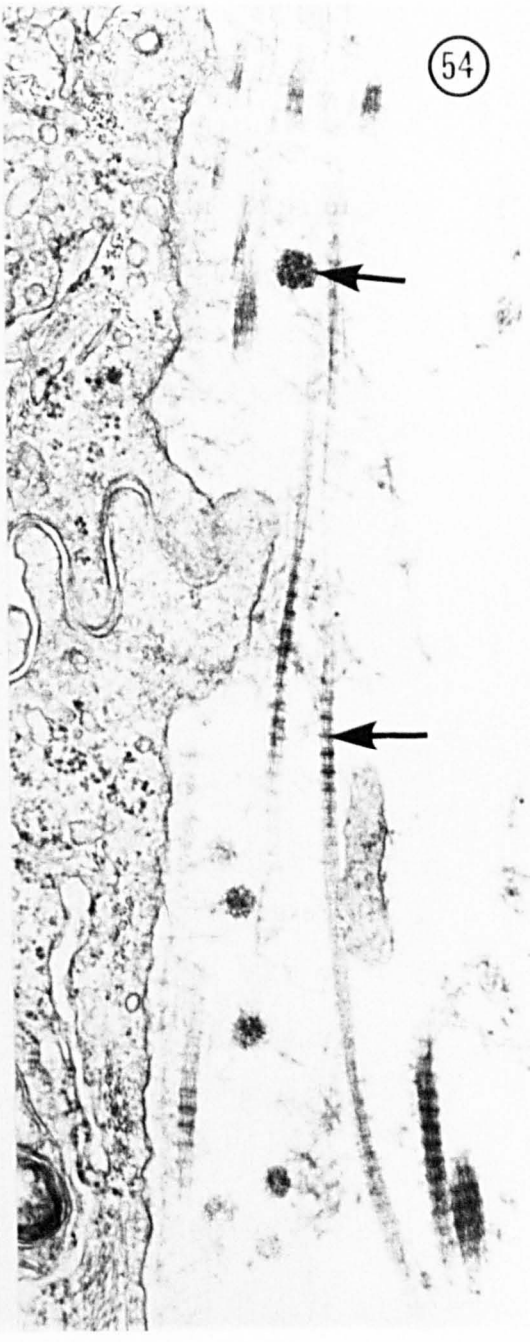
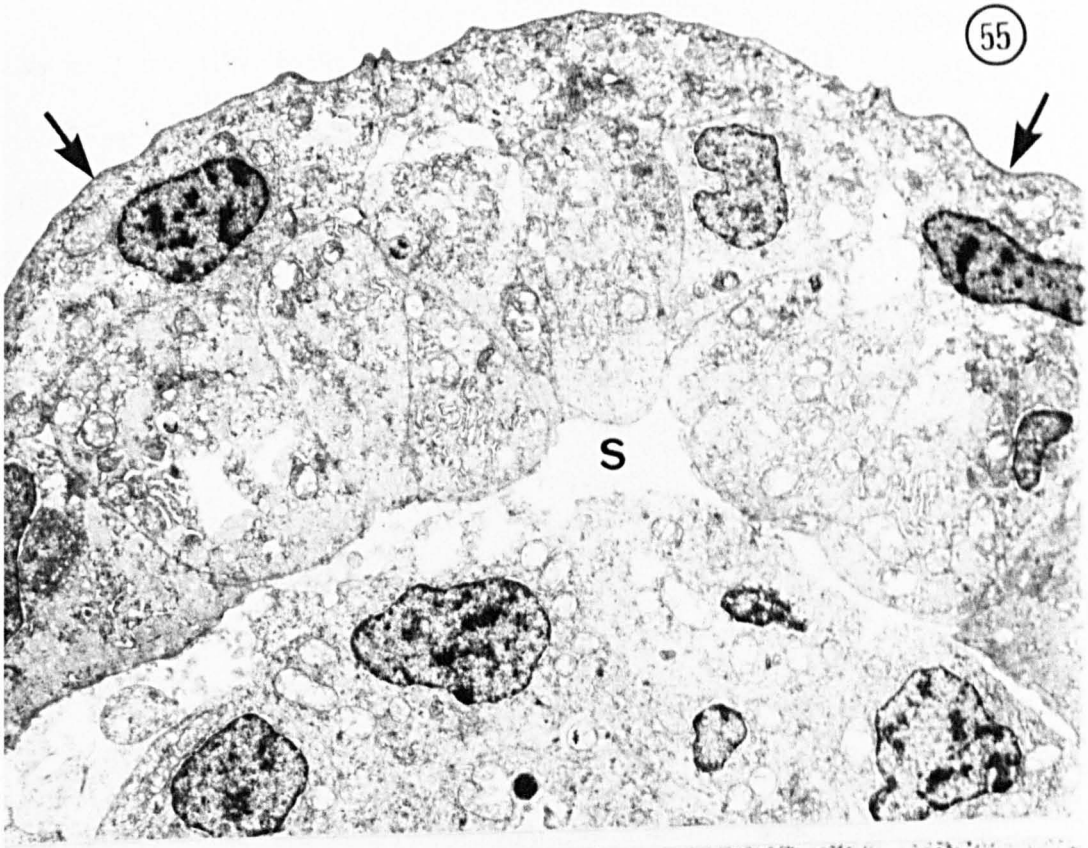

54

Fig. 55

Cross section through a portion of the fin fold ectoderm from a tail bud which had been exposed to tunicamycin for 6 hours throughout the initial phases of apical ectodermal ridge construction and its conversion to an early fin fold. An irregularly shaped extracellular space (S) has formed beneath an abnormal apical ectodermal ridge (between arrows). x 5700.

Fig. 56

Cross section through a portion of the fin fold ectoderm from a tail bud which had been exposed to tunicamycin for 10 hours from the start of apical ectodermal ridge construction. The subepidermal space is bounded by numerous invaginations (arrowed) which are located along the basal surfaces of putative fin fold epidermal cells. x 12500.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S

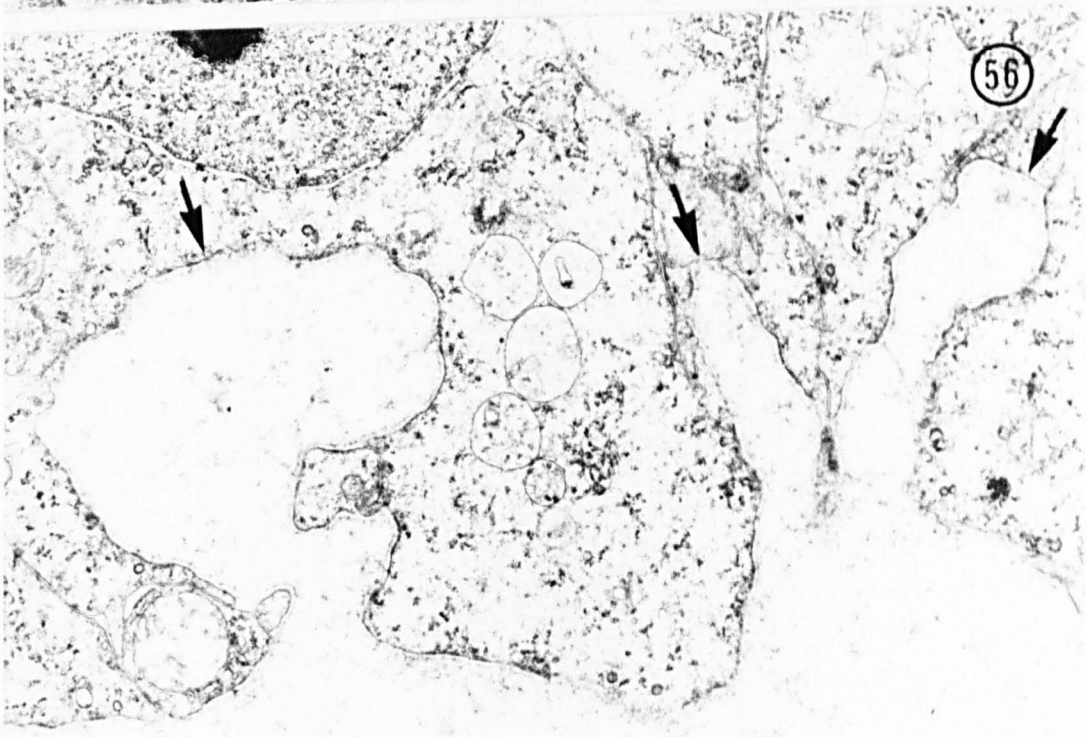

56

Fig. 57

Cross section through the proximal portion of a subepidermal space similar to the one illustrated in Fig. 56 showing a disorganised array of actinotrichia (arrows) within the subepidermal space. x 7500.

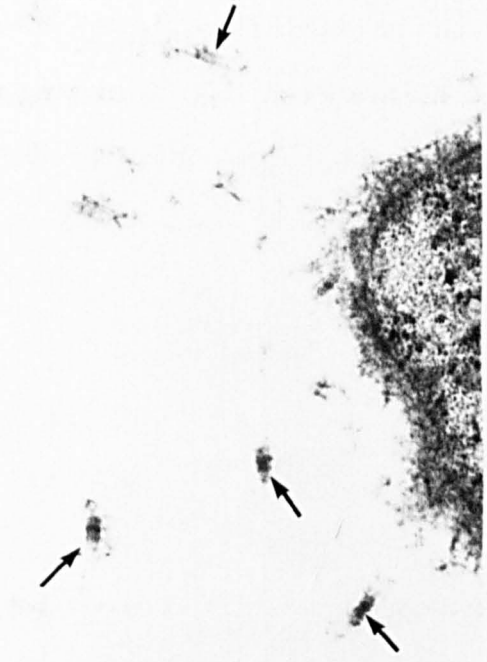
Fig. 58

Methylene blue stained cross section through part of a tail bud which had been exposed to tunicamycin for 10 hours as described for Fig. 56 and then left to recover for 18 hours. An enlarged subepidermal space (S) is located between the fin fold ectoderm (E) and underlying mesoderm (M). x 1560.

Fig. 59

Cross section through a distal portion of the fin fold illustrated in Fig. 58. The subepidermal space (S) is enlarged and contains granular extracellular matrix material. x 10000.

57



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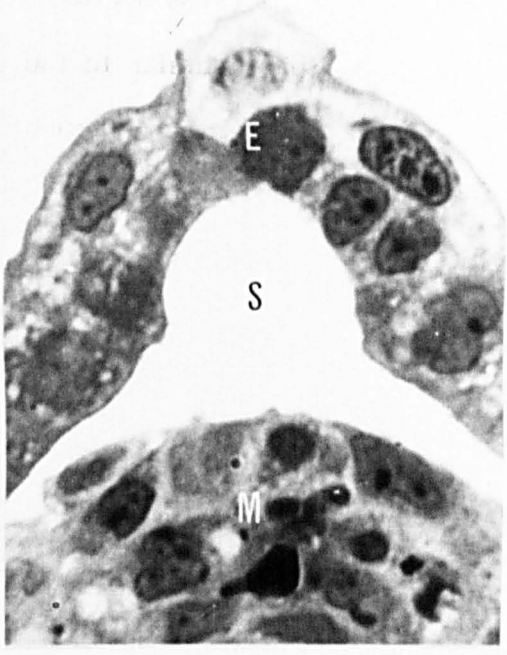

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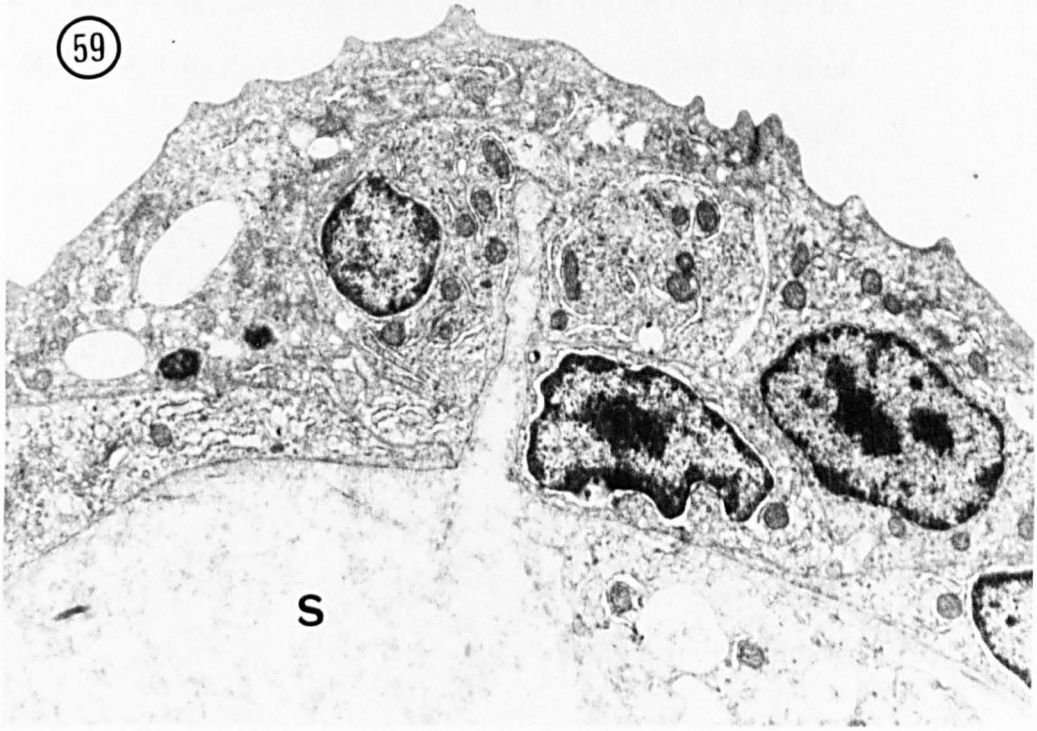


Fig. 60

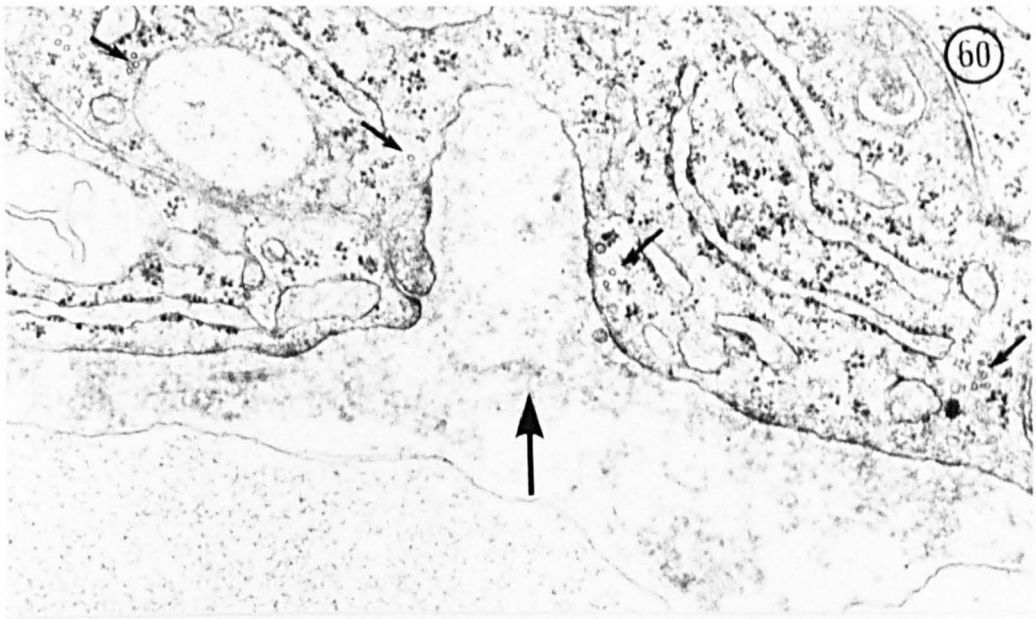
Cross section through a distal portion of a fin fold similar to that illustrated in Fig. 58. Granular matrix material (large arrows) within the subepidermal space extends into the cleft cell-like invagination. Numerous cross-sectional profiles of microtubules are located within the cleft cell cytoplasm (small arrows). x 42000.

Fig. 61

Cross section through a proximal portion of a fin fold similar to that illustrated in Fig. 58. The subepidermal space contains disorganised arrays of actinotrichia (arrows) and granular matrix. x 16500.

Fig. 62

Cross section through the putative fin fold ectoderm from a tail bud which had been exposed to tunicamycin for 35 hours throughout the period of apical ectodermal ridge construction and fin fold development, and during the start of the phase of fin fold expansion. A fin fold is not present and epidermal cells (E) form a flattened layer between the overlying peridermis (P) and underlying mesoderm (M). x 37000.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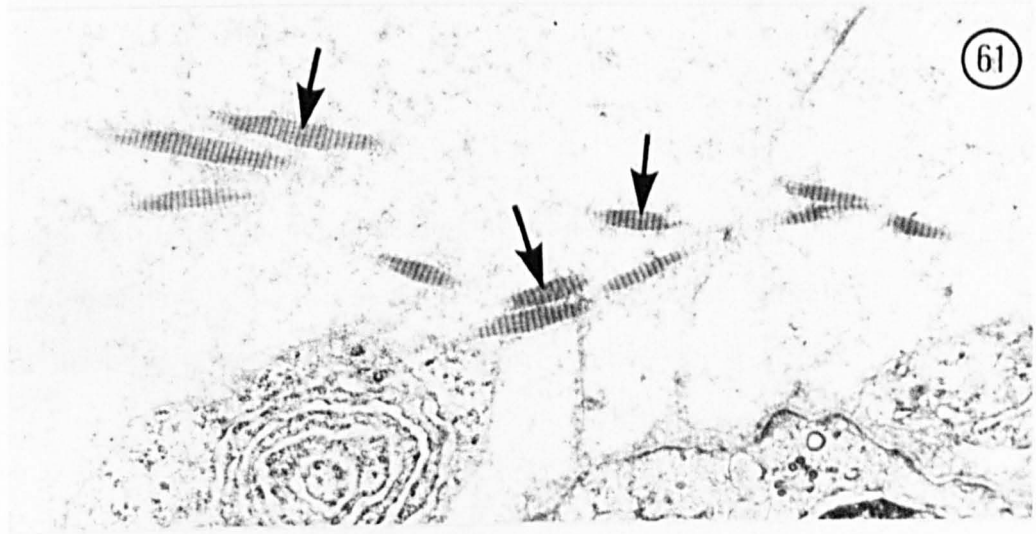

61

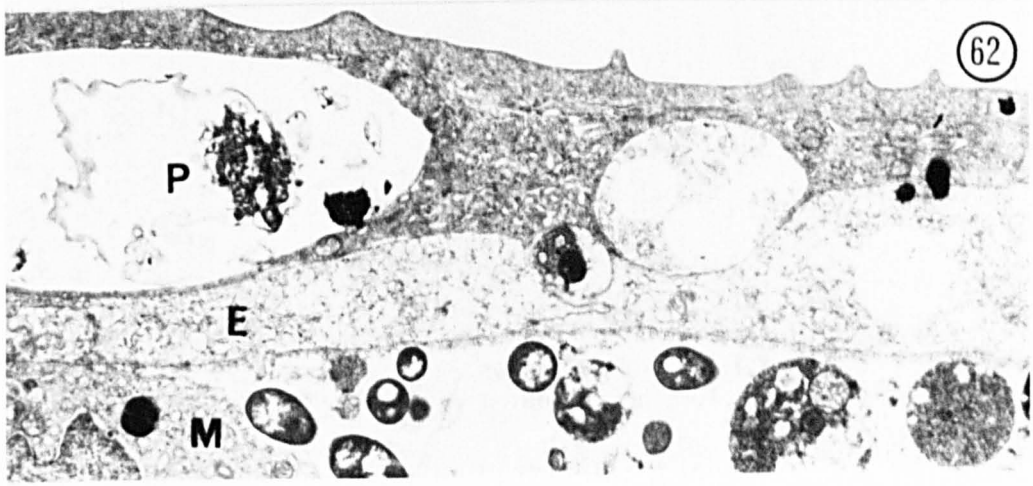

62
P
E
M

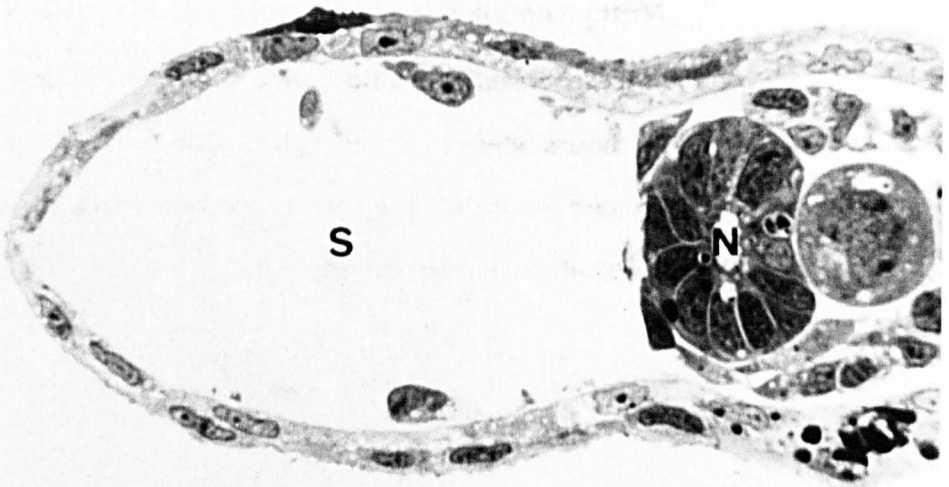
Fig. 63

Methylene blue stained cross section through part of a tail bud and associated fin fold which had been exposed to tunicamycin for 12 hours during stage 21. The subepidermal space (S) is swollen and contains migratory mesodermal cells. The neural tube (N) is well differentiated. x 1350.

Fig. 64

Cross section through an apical portion of the fin fold illustrated in Fig. 63 showing an apical cleft cell (C) and underlying subepidermal space which contains granular extracellular matrix (large arrow) and aligned actinotrichia (small arrows). x 22000.

63



64

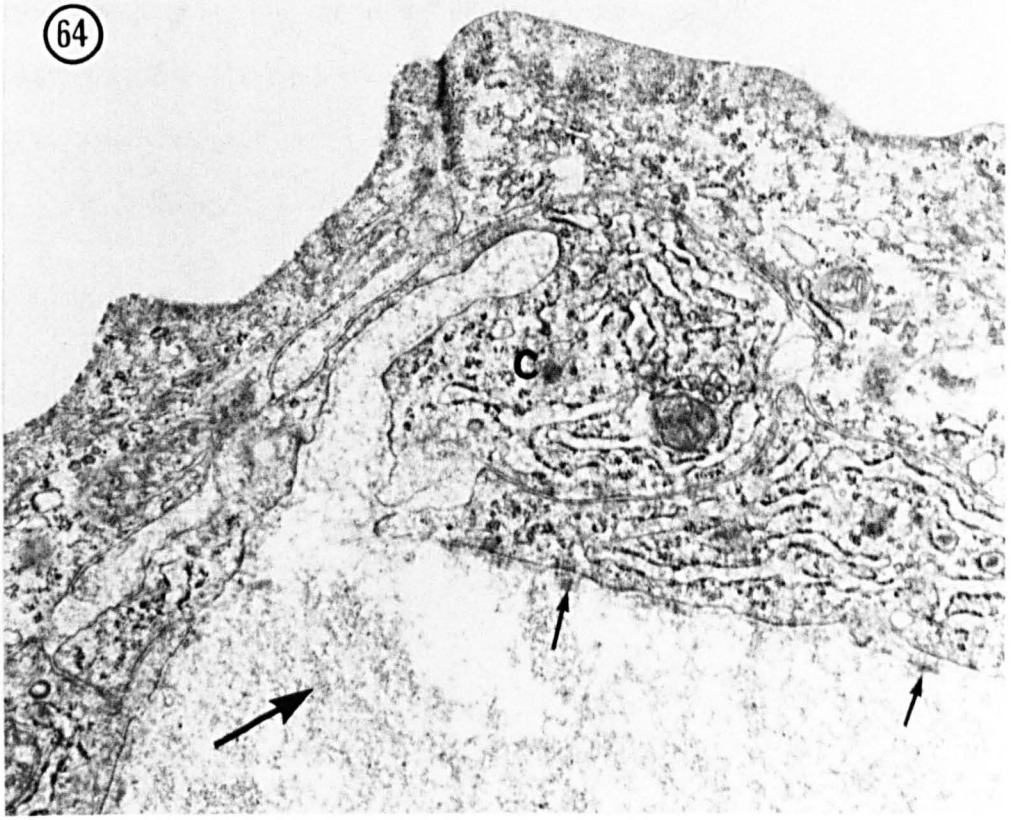
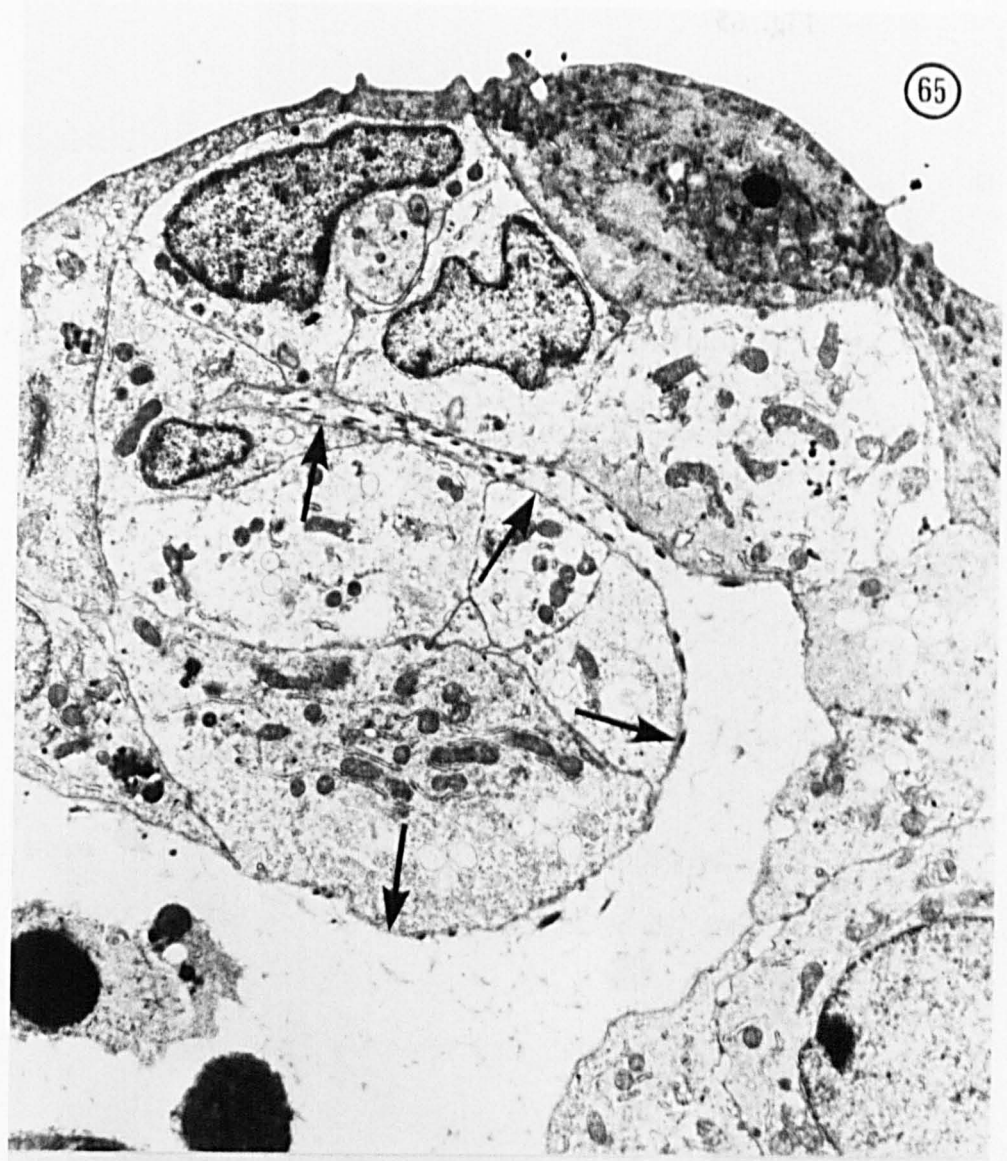


Fig. 65

Cross section through a fin fold from a tail bud which had been exposed to nocodazole throughout fin fold morphogenesis and during the stage of fin fold expansion. Nocodazole prevents the fold from expanding and causes it to collapse laterally: this generates a subepidermal space with a curved cross sectional profile (arrows). x 6300.

Fig. 66

Longitudinal section through the middle of a fold similar to that illustrated in Fig. 65 and cut at right angles to the plane of the fold. The contorted subepidermal space (S) contains numerous profiles of disorganised actinotrichia. x 10400.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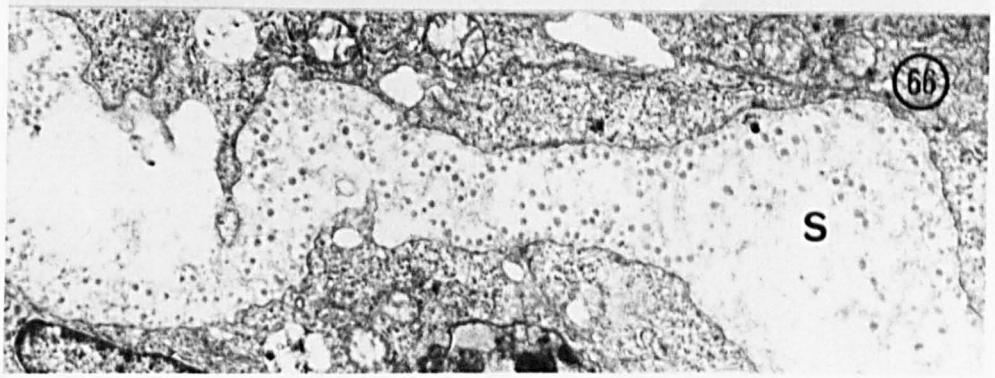
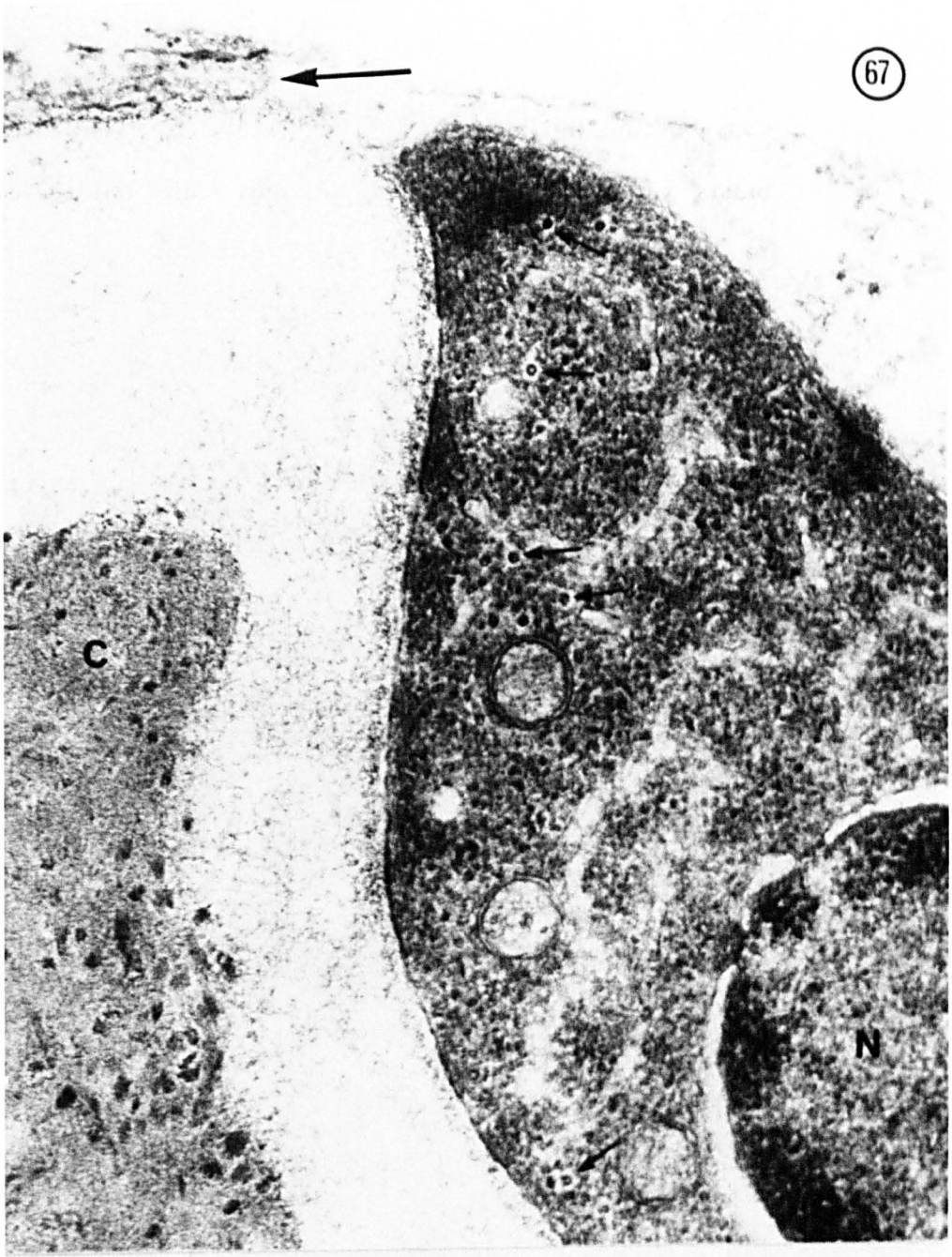

66
S

Fig. 67

Section through part of the osseous layer and layer of osteoblasts of a decalcified Brachydanio scale cut at right angles to the plane of the scale and longitudinal axis of the circuli. The steep posterior edge of a circulus (C) is towards the left of the margin. The anterior edge of the osteoblast is adjacent to the posterior margin of an adjacent osteoblast (large arrow) which runs along the crest of the circulus. Cross sectional profiles of microtubules (small arrows) are located in the cytoplasm between the osteoblast cell surface and the nucleus (N). x 68000.

Fig. 68

Cross section through part of the fibrillary plate of a decalcified Brachydanio scale cut in the same plane as that described for Fig. 67 showing the orthogonal arrangement of extracellular collagenous fibres. The plane of the scale is oriented parallel to the sides of the page. x 25600.


67
C
N

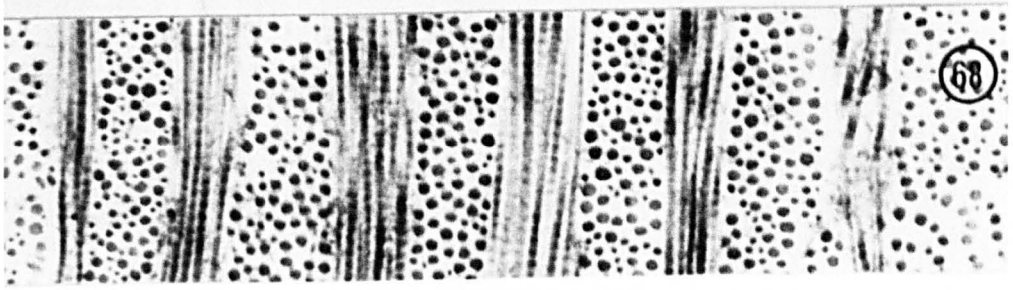
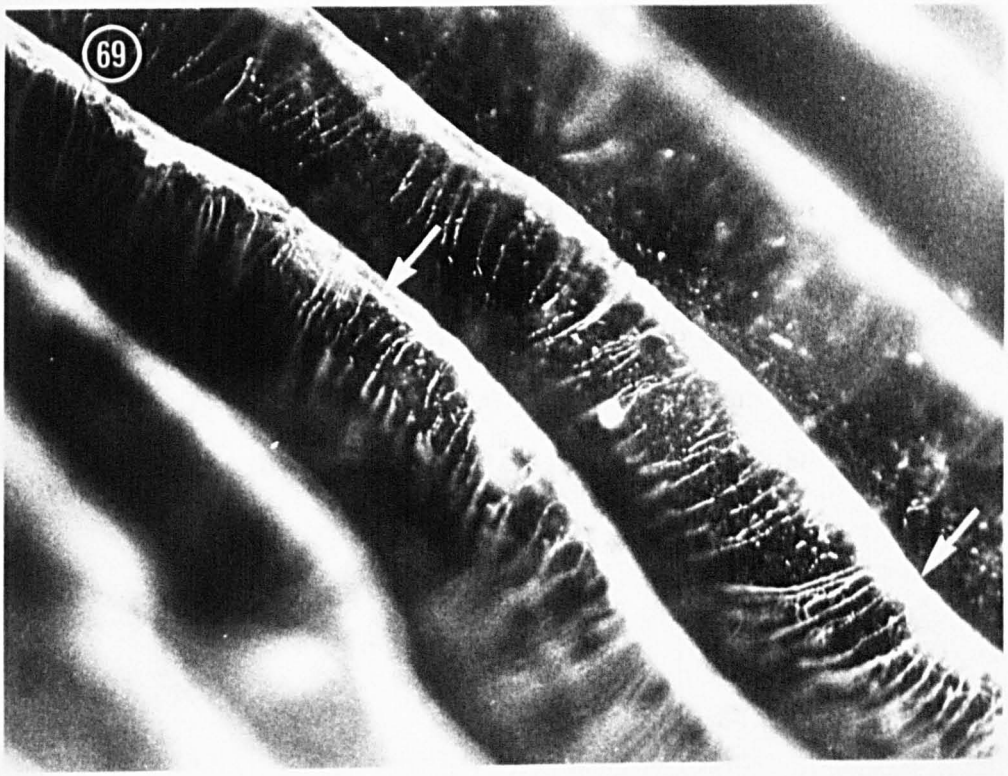

68

Fig. 69

Fluorescence micrograph of part of the osteoblast layer near the anterior edge of a Brachydanio scale. Circuli are oriented across the micrograph (from top left to bottom right). Anterior portions of fluorescently labelled microtubules are oriented parallel to circuli (arrows), whilst posterior portions are oriented more or less at right angles to circuli. x 1025.

Fig. 70

Part of the osteoblast layer from a Brachydanio scale oriented in a similar plane to that described for Fig. 69. In this region circuli are more widely spaced than in Fig. 69 and fluorescently stained microtubules are not as precisely aligned at right angles to circuli. x 1025.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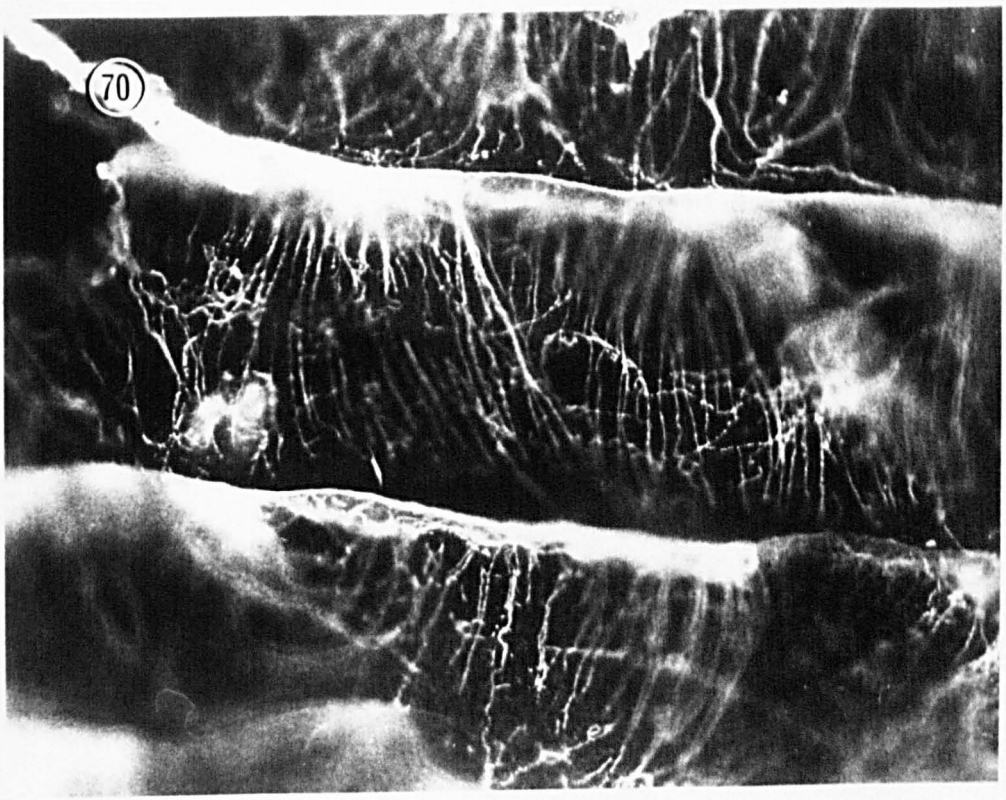
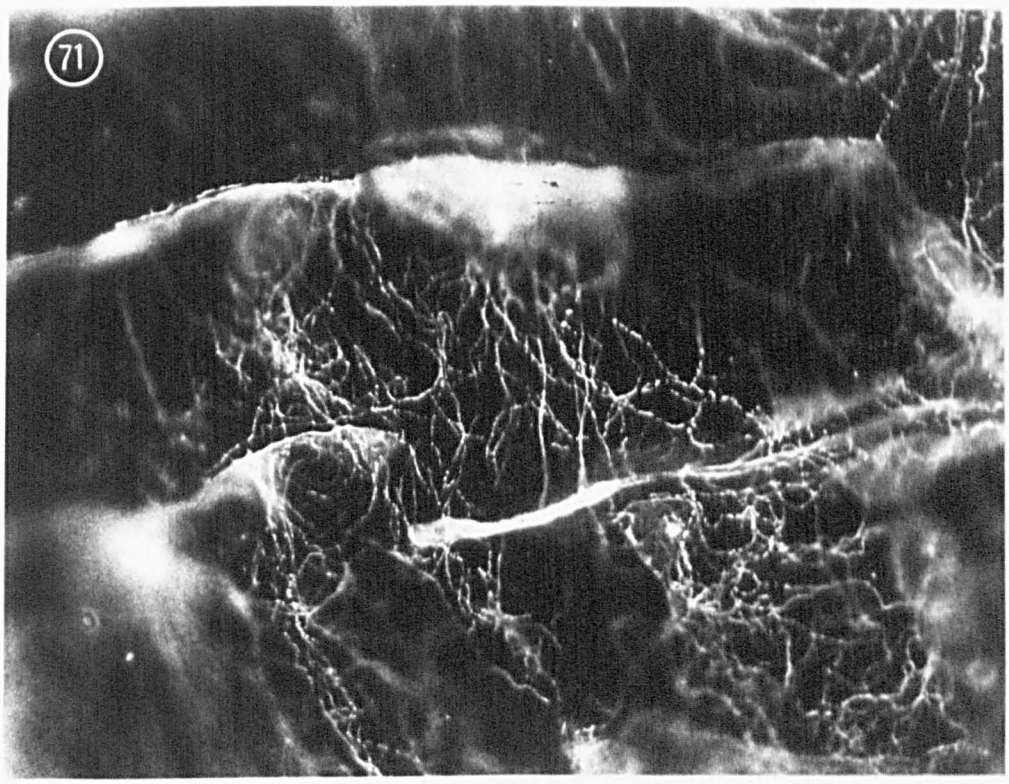

70

Fig. 71

Part of the osteoblast layer of a Brachydanio scale oriented in a similar plane to that described for Fig. 69. In this region circuli are widely spaced and show discontinuities. The organisation of fluorescently stained microtubules only approximates to that shown in Figs. 69 and 70. x 1025.

Fig. 72

Part of the osteoblast layer of a Brachydanio scale oriented in a similar plane to that described for Fig. 69. In this region radii are present (arrows) and fluorescently stained microtubules radiate from a central nuclear region (N). x 1025.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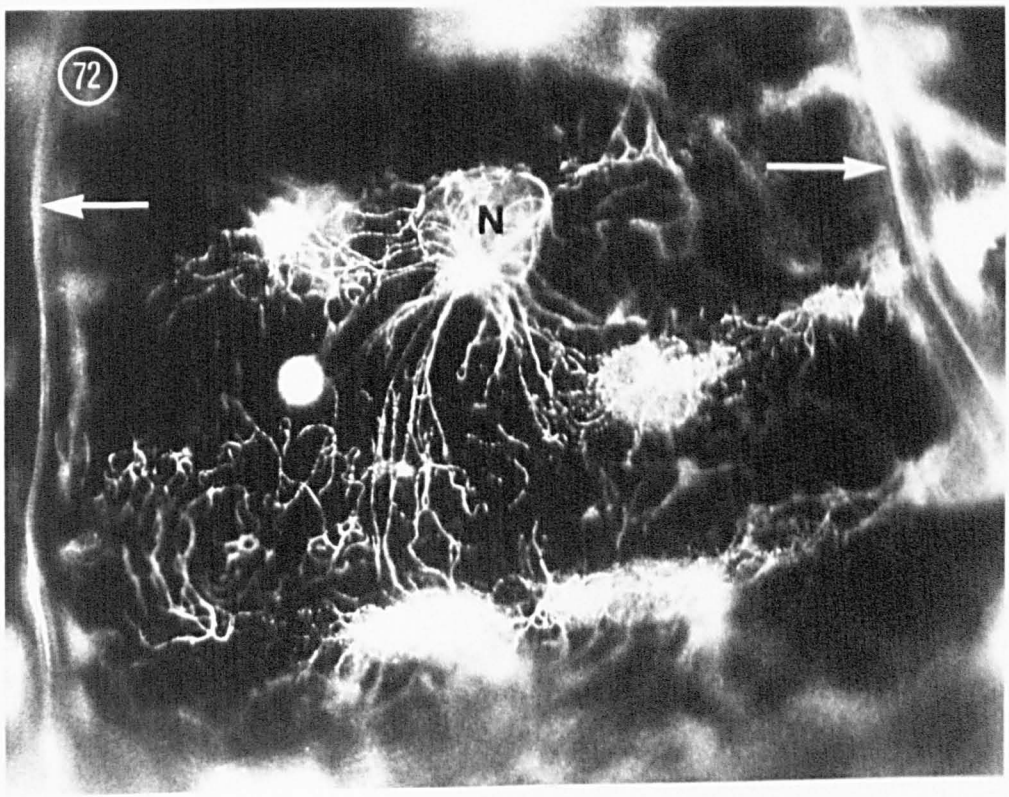
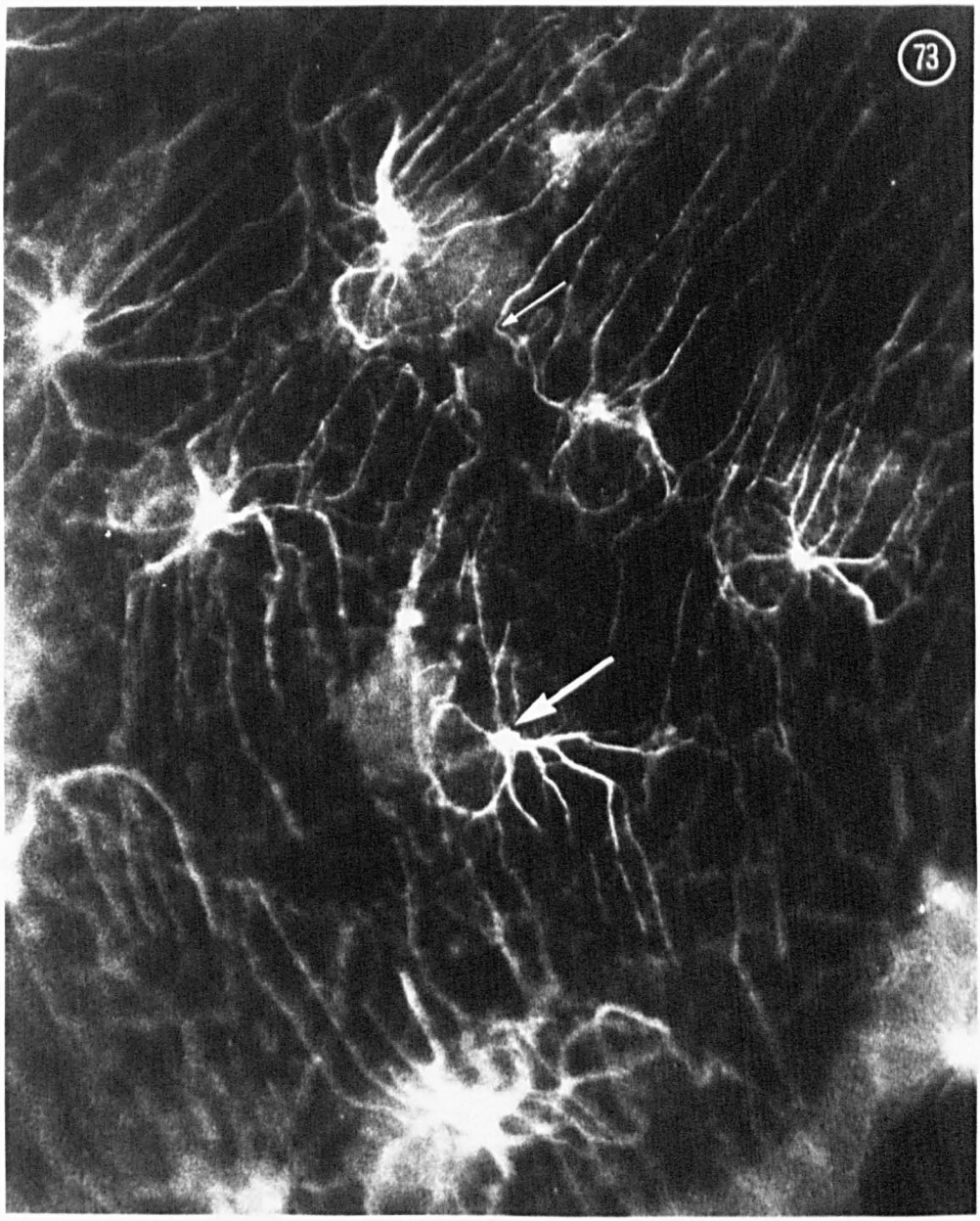

72
N

Fig. 73

Part of the fibroblast layer of a Brachydanio scale showing the arrangement of fluorescently stained microtubules which radiate from a central site (large arrow), portions of these microtubules curve (small arrow) into alignment with each other and with similar portions in neighbouring cells. x 1320.

Fig. 74

Section cut at right angles to the plane of a scale from Brachydanio through the fibrillary plate and associated fibroblast layer showing the thin peripheral portions of two adjacent fibroblasts (arrow). The thickness of the fibroblast layer is not much greater than the spot desmosome and associated filament bundles. Part of the fibrillary plate is shown at the top of the micrograph. x 55000.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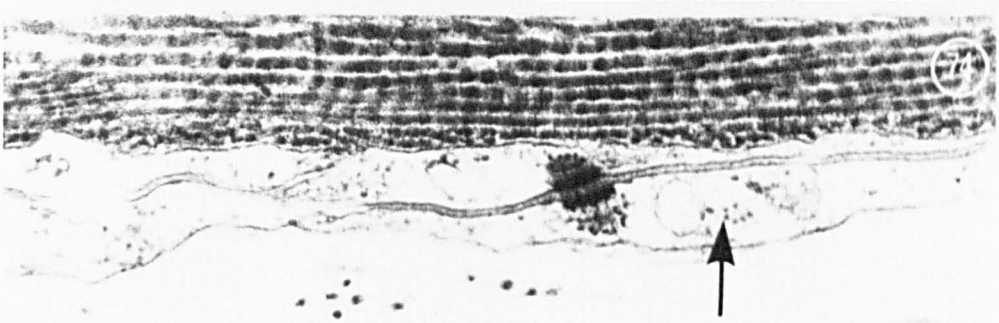
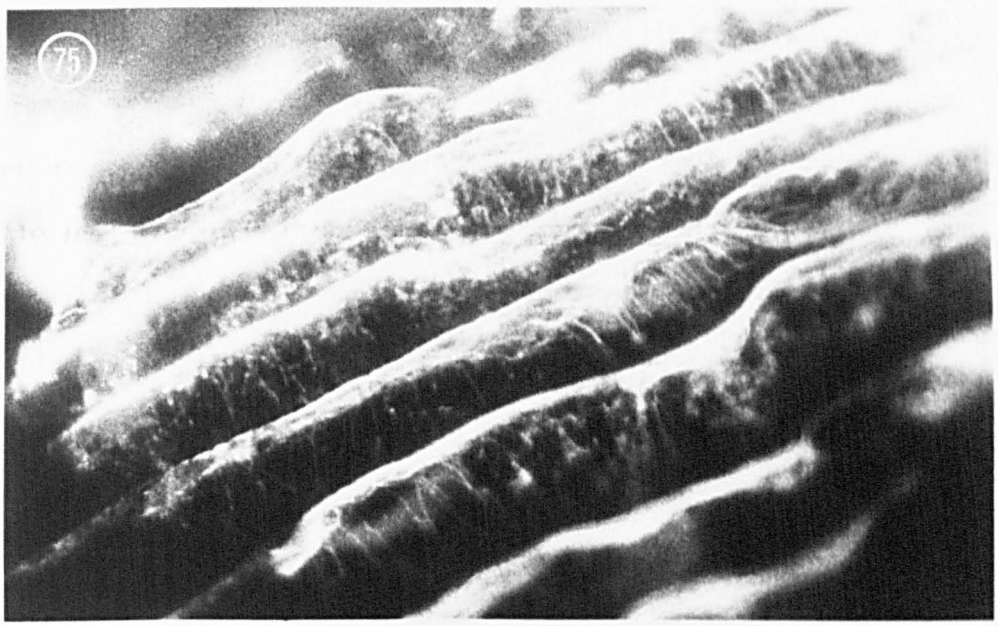

74

Fig. 75

Part of the osteoblast layer near the anterior edge of a Hyphessobrycon scale showing the arrangement of fluorescently stained microtubules which are oriented at right angles to the closely spaced circuli (circuli run from top right to bottom left). The anterior margin of the scale is positioned at the top of the micrograph. x 1100.

Fig. 76

Part of Hyphessobrycon osteoblast layer in a similar orientation to that described for Fig. 75. In this region circuli are more widely spaced and fluorescently stained microtubules curve away from circuli at a wider range of angles than those shown in Fig. 75. x 1100.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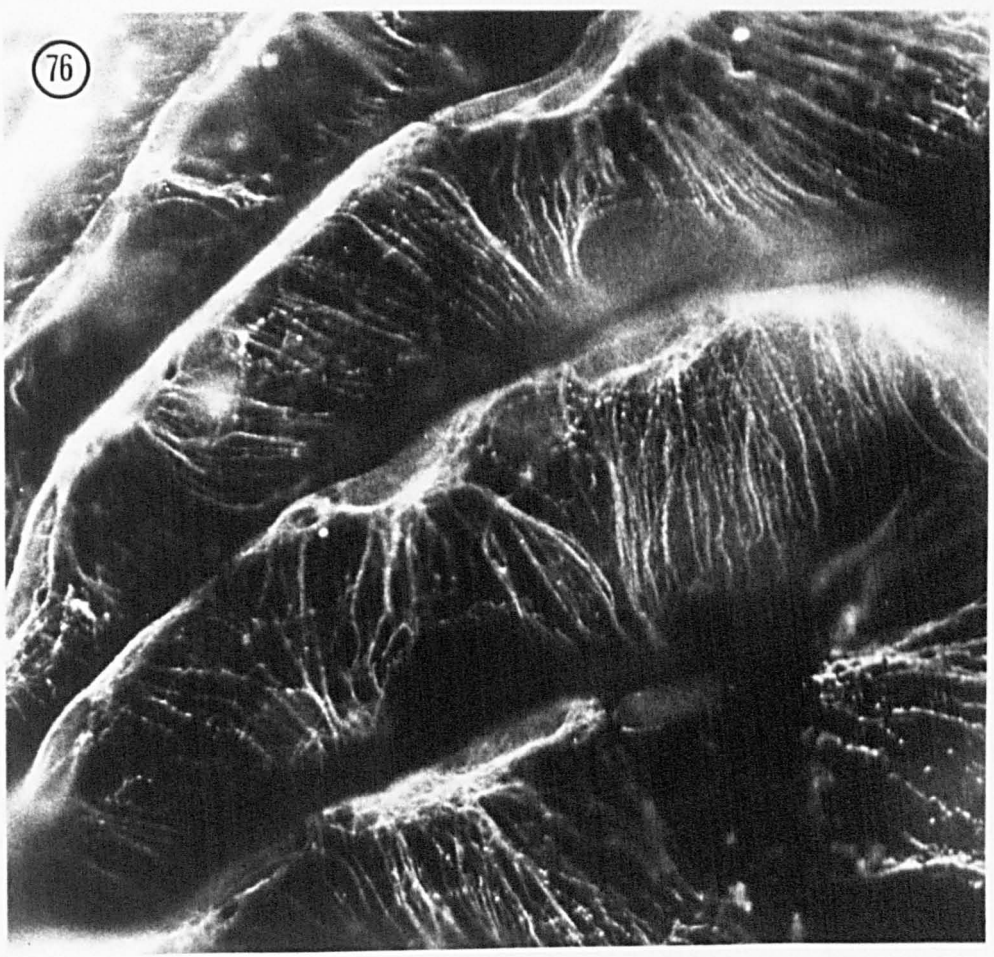

76

Figs. 77 and 78

Part of the osteoblast layer of a Hyphessobrycon scale. In this region radii occur. Microtubules radiate from a central nuclear region (N) and curve (arrow) to contribute to a "marginal band" of microtubules at the periphery of the cell.

Fig. 77 x 1300.

Fig. 78 x 1300.

Fig. 79

Part of a Hyphessobrycon osteoblast from a region identical to that described for Fig. 76 showing curved portions of microtubules (arrow) which run between the portions that are aligned alongside a circulus (towards the top of the micrograph) and those that are aligned at right angles to the circulus, x 3300.

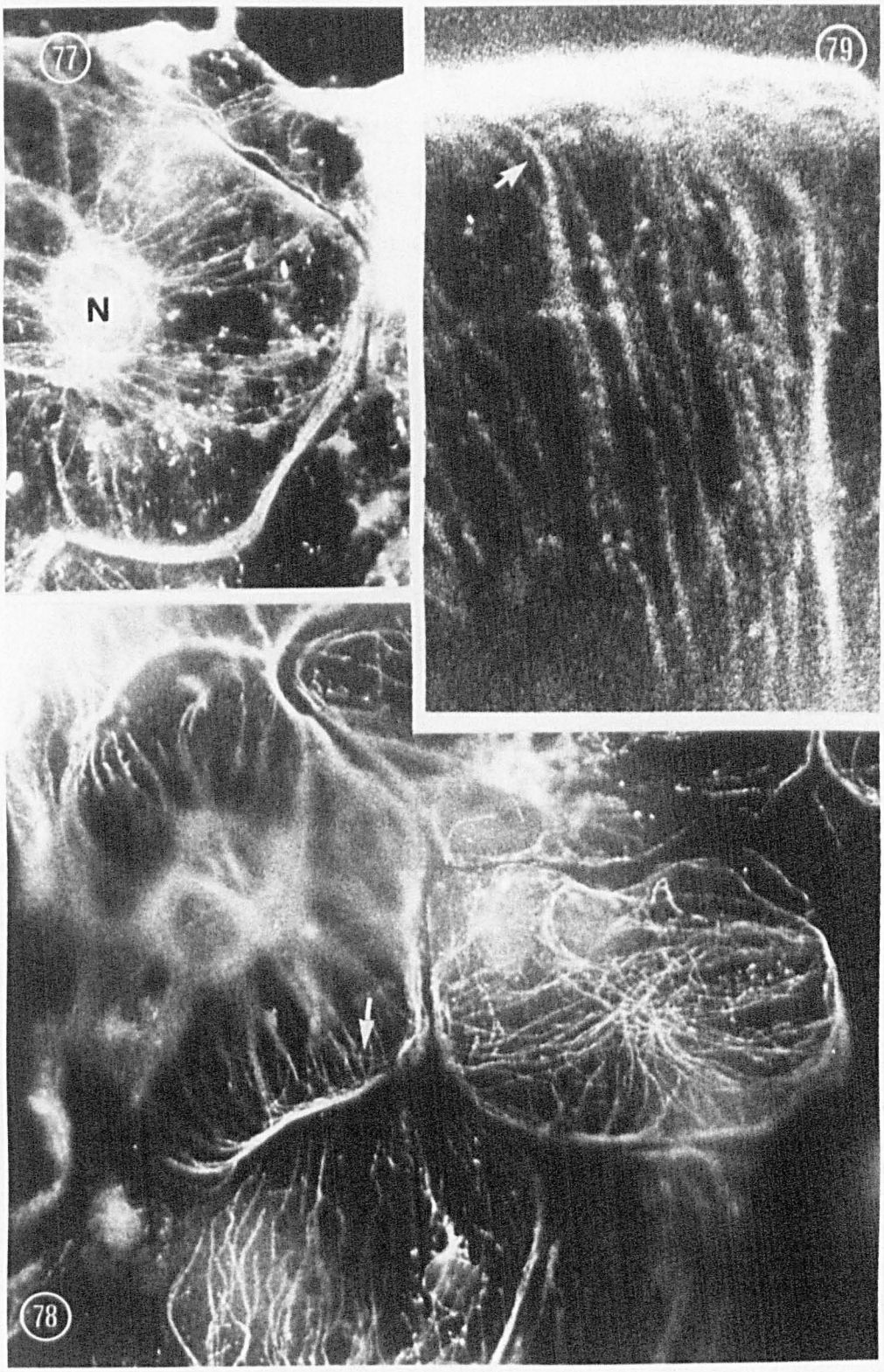
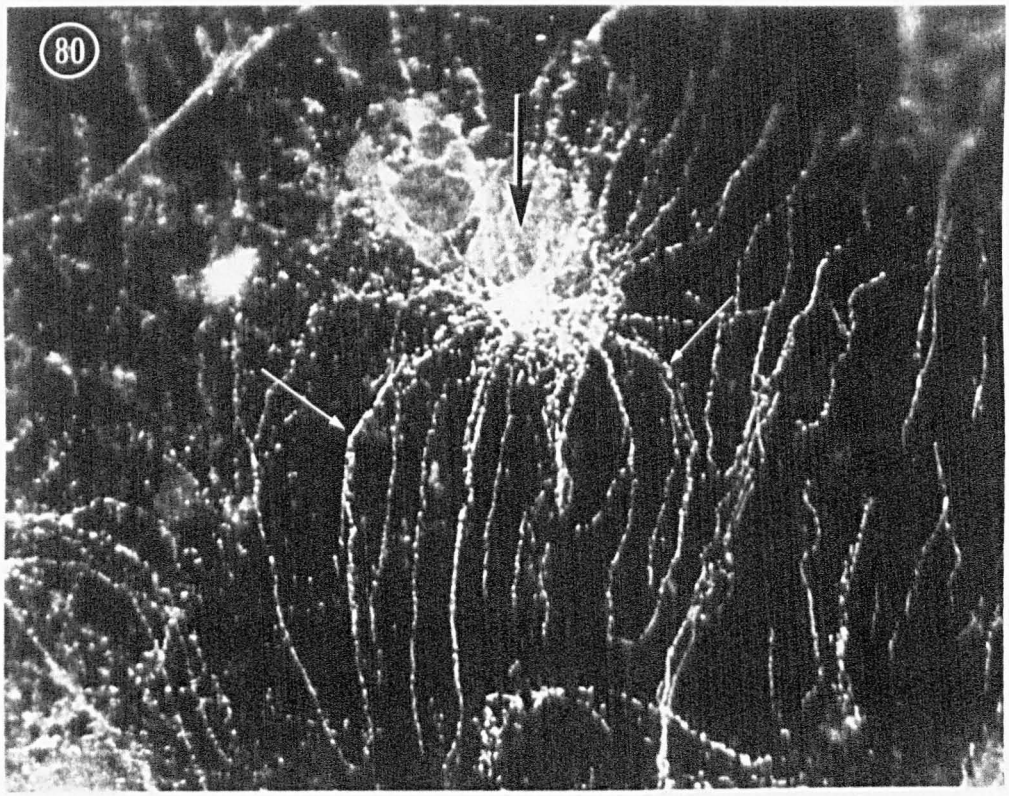

77
N
79
78

Fig. 80

Part of the fibroblast cell layer from a Hyphessobrycon scale showing the arrangement of fluorescently stained microtubules which radiate from a central nuclear region (large arrow) and curve (small arrows) into alignment with adjacent portions of other microtubules in a similar pattern to that described for Brachydanio in Fig. 73. x 1150.

Fig. 81

Part of an osteoblast layer from a Salmo scale. Microtubule arrays are less clear than those of Hyphessobrycon and Brachydanio. Diffusely stained nuclear regions (N) are located between circuli which curve from top right to bottom left of the micrograph. x 1230.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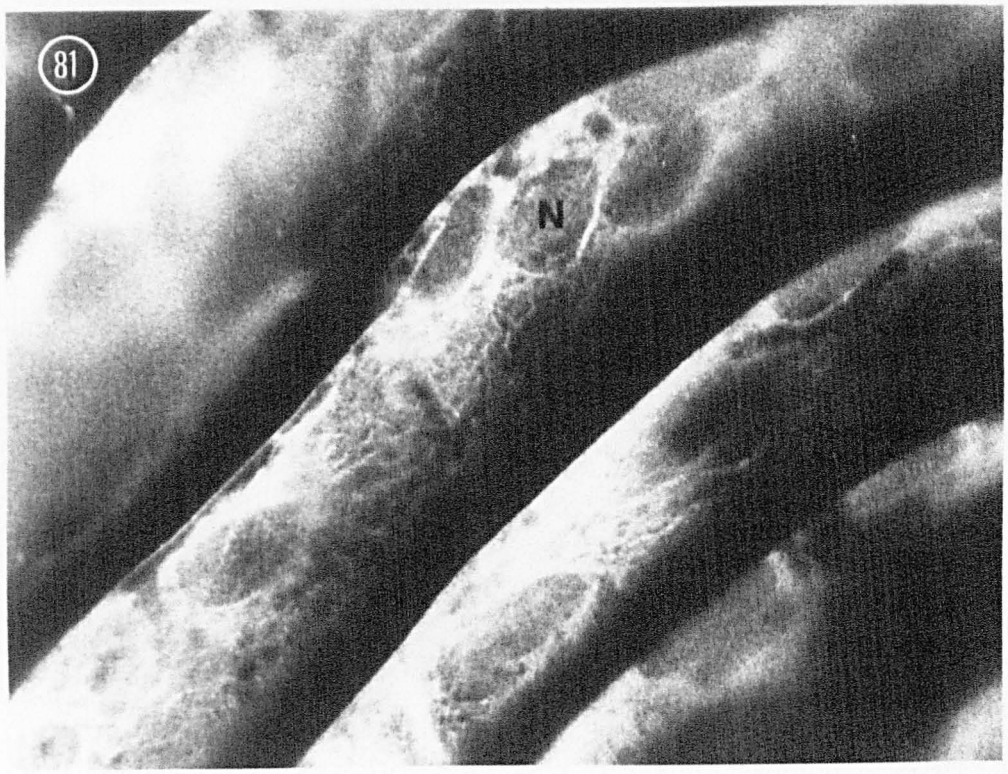
81
N

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